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Award Number: DAMD17-96-1-6199

TITLE: Function of the Alpha6 in Breast Carcinoma

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REPORT DATE: October 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20010716 084

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2000	Annual (20 Sep 99 - 19 Sep 00)

4. TITLE AND SUBTITLE
Function of the Alpha6 in Breast Carcinoma

5. FUNDING NUMBERS
DAMD17-96-1-6199

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Breast carcinoma invasion is a complex process that involves directed migration and localized proteolysis, as well as the ability of breast tumor cells to survive. The research accomplished during Year 4 of this grant has yielded considerable insight into the mechanisms that underlie the survival and migration of breast carcinoma cells. A key component of these mechanisms is the integrin $\alpha 6\beta 4$. Expression of this integrin has been linked to the progression of breast cancer and correlated with poor prognosis. At a mechanistic level, our work has established that this integrin regulates critical signaling pathways involved in both survival and migration that substantiate its involvement in breast cancer progression. These pathways are the PI3-K/Akt pathway, as well pathways involving the Rho GTPases. Importantly, our work has shown that $\alpha 6\beta 4$ can function in concert with growth factor receptors implicated in breast cancer progression such as erBb2. As stated in previous progress reports, elucidation of such pathways provides specific targets for therapeutic intervention.

14. SUBJECT TERMS
Breast Cancer, Invasion, Integrins, Signaling

15. NUMBER OF PAGES
48

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover..... 1

SF 298..... 2

Table of Contents..... 3

Introduction..... 4

Body..... 4

Key Research Accomplishments..... 5

Reportable Outcomes..... 6

Conclusions..... 7

References..... 7

Appendices..... 8

5) INTRODUCTION:

Breast carcinoma invasion is a complex process that involves directed migration and localized proteolysis, as well as the ability of breast tumor cells to survive (1). Although the mechanistic basis of invasion had been elusive, recent advances in molecular cell biology have facilitated a much more rigorous analysis of this important and critical component of cancer progression. In particular, insight into the function and regulation of cell adhesion receptors, as well as proteases, has fueled significant progress in our understanding of the invasive process. Studies aimed at defining specific signal transduction pathways that determine the behavior of invasive carcinoma cells are also contributing to an uncovering of the molecular basis of invasion. Recent work by our group and others has implicated a key role for the α6β4 integrin in breast carcinoma invasion (2-7). This dynamic function of α6β4 in enhancing the migration of invasive carcinoma cells is quite distinct from its role in maintaining stable adhesive contacts in normal breast epithelia by associating with intermediate filaments. In fact, we have established that the ability of α6β4 to stimulate breast carcinoma migration and invasion depends upon its preferential activation of a PI3-K/Rac signaling pathway that is necessary for invasion (6). In essence, our studies have defined an integrin-mediated mechanism of breast carcinoma invasion that involves the stimulation of carcinoma migration by the dynamic association of α6β4 with F-actin and the activation of a specific signaling pathway by this integrin.

Studies carried out during Year 4 of this grant have extended our analysis of the contribution of the α6β4 integrin, as well as other integrins, to breast carcinoma progression significantly. The major findings of Year 4 are summarized below and reprints of Year 4 publications are included in the Appendix.

6) BODY

The α6β4 integrin promotes the survival of p53-deficient breast carcinoma cells: Although the interaction of matrix proteins with integrins is known to initiate signaling pathways that are essential for cell survival, a role for tumor suppressors in the regulation of these pathways has not been established. We demonstrate here that p53 can inhibit the survival function of integrins by inducing the caspase-dependent cleavage and inactivation of the serine/threonine kinase AKT/PKB. Specifically, we show that the α6β4 integrin promotes the survival of p53-deficient breast carcinoma cells by activating AKT/PKB. In contrast, this integrin does not activate AKT/PKB in carcinoma cells that express wild-type p53 and it actually stimulates their apoptosis, in agreement with our previous findings (Bachelder et al. 1999. JBC 274:20733-20737). Interestingly, we observed reduced levels of AKT/PKB protein following antibody clustering of α6β4 in carcinoma cells that express wild-type p53. In contrast, α6β4 clustering did not reduce the level of AKT/PKB in carcinoma cells that lack functional p53. The involvement of caspase 3 in AKT/PKB regulation was indicated by the ability of Z-DEVD-FMK, a caspase 3 inhibitor, to block the α6β4-associated reduction in AKT/PKB levels *in vivo*, and by the ability of recombinant caspase 3 to promote the cleavage of AKT/PKB *in vitro*. In addition, the ability of α6β4 to activate AKT/PKB can be restored in p53-wild-type carcinoma cells by inhibiting caspase 3 activity. These studies demonstrate that the p53 status of an α6β4-expressing breast carcinoma cell influences its growth and survival potential. See Bachelder et al, J. Cell Biology, 147:1063-1072 (Appendix). These research accomplishments are associated with Technical Objectives 2 and 4 of the original proposal.

Cooperative signaling between α6β4 integrin and ErbB2 receptor is required to promote PI3-kinase-dependent invasion. In this collaborative study, evidence was provided that the a6b4 integrin functions in concert with erbB2, a EGF family receptor linked to breast cancer progression, to promote activation of PI3-K and invasion. These findings are published in the Gambaletta et al. **J. Biological Chemistry**, 275:10604-10610 (Appendix).

RhoA function in lamellae formation and migration of carcinoma cells is regulated by the α6β4 integrin and cAMP metabolism: The integrin α6β4 promotes the formation of actin-rich protrusions and stimulates the migration of breast carcinoma cells. In this study, we examined the involvement of RhoA in these events and the regulation of RhoA activation by integrin signaling. Clone A carcinoma cells develop fan-shaped lamellae and exhibit random migration when plated on laminin, processes that depend on α6β4. Here, we report that expression of a dominant negative RhoA (N19RhoA) in clone A cells inhibited α6β4-dependent membrane ruffling and the formation of lamellae by 80%. Inhibition of RhoA also blocked the migration of clone A cells but had minimal effects on spreading. Using the Rhotekin binding assay to assess RhoA activation, we observed that engagement of α6β4 by either laminin or antibody-mediated clustering resulted in a 3-4 fold greater increase in RhoA activation than engagement of β1 integrins. The α6β4-mediated interaction of clone A cells with laminin promoted the translocation of RhoA from the cytosol to membrane ruffles at the edges of lamellae and promoted its co-localization with β1 integrins, as assessed by immunofluorescence microscopy. In addition, RhoA translocation was blocked by inhibiting phosphodiesterase activity and enhanced by inhibiting the activity of cAMP-dependent protein kinase A. Together, these results establish a specific integrin-mediated pathway of RhoA activation that is regulated by cAMP and that functions in lamellae formation and migration. NOTE: The data to support these conclusions can be found in the O'Connor et al. J. Cell Biology 148:253-258 (Appendix). These research accomplishments are associated with Technical Objectives 2 and 4 of the original proposal.

The migration of breast carcinoma cells requires both cAMP-phosphodiestetase and cAMP-dependent protein kinase A activity. Increases in the intracellular concentration of cAMP ($[cAMP]_i$) stimulate the activity of protein kinase A (PKA) and impede the migration of carcinoma cells by a mechanism that involves PKA phosphorylation of Rho A (O'Connor et al, *J. Cell Biol.* 148:253-258; Lang et al., *EMBO J.* 15:510-519). Phosphodiesterases (PDEs) are necessary for migration because they prevent this cAMP inhibition of RhoA (O'Connor et al, *J. Cell Biol.* 148:253-258). We report here that the chemotactic migration of breast carcinoma cells involves not only PDE activity but also a PKA-dependent signaling event involving Rac1. Importantly, we identify β1 integrin signaling as a stimulus of PKA activity in migrating cells and demonstrate that the activation of Rac by β1 integrins requires PKA activity. A novel implication of these findings is that PKA can differentially regulate the activity and function of Rac and Rho and it may mediate a spatial and temporal regulation of these GTPases during migration. NOTE: The data to support these conclusions can be found in the O'Connor and Mercurio manuscript provided in the Appendix. These research accomplishments are associated with Technical Objectives 2 and 4 of the original proposal.

Contractual Issues: This award is currently in a no-cost extension period for completion of Objectives 3 and 4.

7) KEY RESEARCH ACCOMPLISHMENTS:

- The α6β4 integrin activates the phosphoinositide 3-OH kinase/AKT survival pathway in p53-deficient breast carcinoma cells.
- In p53-wild-type carcinoma cells, a caspase-3 mediated cleavage of AKT kinase occurs that inhibits the α6β4-mediated survival pathway.
- The α6β4 integrin functions in concert with erbB2 to activate PI3-K and promote invasion.
- Both the RhoA and Rac1 GTPases are essential for the migration and invasion of breast carcinoma cells.

- cAMP inhibits the migration and invasion of breast carcinoma cells by preventing Rho A activation
- PKA is necessary for the migration of breast carcinoma cells
- PKA is needed for Rac activation in invasive breast carcinoma cells
- The migration of breast carcinoma cells is controlled by cAMP-dependent, as well as cAMP-inhibitory signaling mechanisms
- An important implication of our findings this year is that localized fluctuations in the $[cAMP]_i$ in breast carcinoma cells may provide a spatial and temporal regulation of PKA activity that influences Rac and RhoA function and determines their ability to migrate and invade
-

8) REPORTABLE OUTCOMES:

Manuscripts:

Bachelder, R., Ribick, M., Marchetti, A., Falcioni, R., Soddu, S., Davis, K. and AM Mercurio. 1999. p53 inhibits α6β4 integrin survival signaling by promoting the caspase-3 dependent cleavage of AKT/PKB. **J. Cell Biology**, 147:1063-1072.

O'Connor, KL, Nguyen, B-K. and AM Mercurio. 2000. RhoA function in lamellae formation and migration is regulated by the α6β4 integrin and cAMP metabolism. **J. Cell Biology**, 148:253-258.

Gamballeta, D., Marchetti, A., Benedetti, L., Mercurio, A.M., Sacchi, A. and R Falcioni. 2000. Cooperative signaling between α6β4 integrin and ErbB2 receptor is required to promote PI3-kinase-dependent invasion. **J. Biological Chemistry**, 275:10604-10610.

O'Connor, KL, Nguyen, B-K. and AM Mercurio. 2000. Integrin-mediated stimulation of protein kinase A regulates Rac and promotes the chemotactic migration of breast carcinoma cells. Submitted manuscript.

O'Connor, K. L., L. M Shaw and A. M. Mercurio. 2000. Integrin α6β4 stimulates chemotactic migration of carcinoma cells by regulating cAMP metabolism. Department of Defense Era of Hope Breast Cancer Meeting, Atlanta, GA.

Mercurio, A.M., Bachelder, R.E., Rabinovitz, I., O'Connor, K.L., and T.T. Tani. 2000. The metastatic odyssey. The integrin connection. **Surgical Oncology Clinics of North America**. (In Press).

Mercurio, A.M. and I. Rabinovitz. 2001. Towards a molecular understanding of tumor invasion-lessons from the α6β4 integrin. **Seminars in Cancer Biology** (In Press).

Major Presentations: (All presentations were focused on the contribution of the α6β4 integrin to breast cancer progression)

- US-Japan Workshop "Molecular Basis of Tumor Invasion and Metastasis:Strategic Applications to Cancer Therapy" (Maui, Hawaii) (February, 2000)
- 24th German Cancer Congress (Berlin, Germany) (March 2000)
- Gordon Conference "Mammary Gland Biology" (Lucca, Italy) (May 2000)
- Department of Medicine, University of Florence, Italy (May, 2000)
- Cancer Center, Massachusetts General Hospital (Boston, MA) (May, 2000)
- FASEB Conference "Rho GTPases" (Snowmass, CO) (July, 2000)
- Italian Society for Cancer Research (Turin, Italy) (October, 2000)
- Istituto Regina Elena (Rome, Italy) (October, 2000)

9) CONCLUSIONS:

The research accomplished during Year 4 of this grant has yielded considerable insight into the mechanisms that underlie the survival and migration of breast carcinoma cells. A key component of these mechanisms is the integrin α6β4. Expression of this integrin has been linked to the progression of breast cancer and correlated with poor prognosis (8). At a mechanistic level, our work has established that this integrin regulates critical signaling pathways involved in both survival and migration that substantiate its involvement in breast cancer progression. These pathways are the PI3-K/Akt pathway, as well pathways involving the Rho GTPases. Importantly, our work has shown that α6β4 can function in concert with growth factor receptors implicated in breast cancer progression such as erBb2. As stated in previous progress reports, elucidation of such pathways provides specific targets for therapeutic intervention.

10) REFERENCES: (Note: Most references are cited in the three manuscripts that are included in the Appendix and they are not duplicated here)

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11) APPENDIX: The following manuscripts are included in the Appendix:

Bachelder, R., Ribick, M., Marchetti, A., Falcioni, R., Soddu, S., Davis, K. and AM Mercurio. 1999. p53 inhibits α6β4 integrin survival signaling by promoting the caspase-3 dependent cleavage of AKT/PKB. **J. Cell Biology**, 147:1063-1072.

O'Connor, KL, Nguyen, B-K. and AM Mercurio. 2000. RhoA function in lamellae formation and migration is regulated by the α6β4 integrin and cAMP metabolism. **J. Cell Biology**, 148:253-258.

Gamballeta, D., Marchetti, A., Benedetti, L., Mercurio, A.M., Sacchi, A. and R Falcioni. 2000. Cooperative signaling between α6β4 integrin and ErbB2 receptor is required to promote PI3-kinase-dependent invasion. **J. Biological Chemistry**, 275:10604-10610.

O'Connor, KL, Nguyen, B-K. and AM Mercurio. 2000. Integrin-mediated stimulation of protein kinase A regulates Rac and promotes the chemotactic migration of breast carcinoma cells. Submitted manuscript.

p53 Inhibits α6β4 Integrin Survival Signaling by Promoting the Caspase 3–dependent Cleavage of AKT/PKB

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Abstract. Although the interaction of matrix proteins with integrins is known to initiate signaling pathways that are essential for cell survival, a role for tumor suppressors in the regulation of these pathways has not been established. We demonstrate here that p53 can inhibit the survival function of integrins by inducing the caspase-dependent cleavage and inactivation of the serine/threonine kinase AKT/PKB. Specifically, we show that the α6β4 integrin promotes the survival of p53-deficient carcinoma cells by activating AKT/PKB. In contrast, this integrin does not activate AKT/PKB in carcinoma cells that express wild-type p53 and it actually stimulates their apoptosis, in agreement with our previous findings (Bachelder, R.E., A. Marchetti, R. Falcioni, S. Soddu, and A.M. Mercurio. 1999. *J. Biol. Chem.* 274:20733–20737). Interestingly, we observed reduced levels of AKT/PKB protein after antibody clustering of α6β4 in carcinoma cells that express wild-type p53. In contrast, α6β4 clustering did not reduce the level of AKT/PKB in carcinoma cells that lack functional p53. The involvement of caspase 3 in AKT/PKB regulation was indicated by the ability of Z-DEVD-FMK, a caspase 3 inhibitor, to block the α6β4-associated reduction in AKT/PKB levels in vivo, and by the ability of recombinant caspase 3 to promote the cleavage of AKT/PKB in vitro. In addition, the ability of α6β4 to activate AKT/PKB could be restored in p53 wild-type carcinoma cells by inhibiting caspase 3 activity. These studies demonstrate that the p53 tumor suppressor can inhibit integrin-associated survival signaling pathways.

Key words: p53 • integrin • AKT/PKB • survival • caspase

PRIMARY epithelial (Frisch and Francis, 1994) and endothelial (Meredith et al., 1993) cells are prone to anoikis, a form of programmed cell death, when grown in the absence of growth factors and extracellular matrix proteins. This default apoptotic pathway is thought to be important in preventing cell growth at inappropriate anatomical sites. Survival signaling pathways associated with both growth factor receptors and cell adhesion molecules are important in protecting cells from anoikis. For example, growth factors such as EGF, PDGF, and insulin can promote the survival of serum-starved epithelial cells (Merlo et al., 1995; Rampalli and Zelenka, 1995; Rodeck et al., 1997). Similarly, the binding of integrins such as αvβ3 (Stromblad et al., 1996), α5β1 (Zhang et al., 1995), and α6β1 (Howlett et al., 1995; Wewer et al., 1997; Farrelly et al., 1999) to the appropriate extracellular matrix protein can inhibit anoikis. These survival signals have been attributed to the ability of integrins to activate numerous molecules including focal adhesion kinase (Frisch et al., 1996), integrin-linked kinase (Radeva et al., 1997), AKT/PKB (Khwaja et al., 1997), and bcl-2 (Zhang et al., 1995; Stromblad et al., 1996). In addition, integrin survival functions have been associated with their ability to inhibit the activity of p53 (Stromblad et al., 1996; Ilic et al., 1998) and Rb (Day et al., 1997) tumor suppressors. Tumor cells acquire a partial resistance to anoikis as a result of their transformation, which is thought to activate select survival signaling pathways in these cells constitutively (Frisch and Francis, 1994). For this reason, the identification of molecules that can inhibit survival signaling is crucial for developing strategies aimed at blocking tumor cell growth.

The α6β4 integrin, a receptor for the laminin family of extracellular matrix proteins, plays an important role in diverse cellular activities. In addition to serving an important structural role in the assembly of hemidesmosomes in epithelial cells (Borradori and Sonnenberg, 1996; Green and Jones, 1996), α6β4 promotes carcinoma cell migration and invasion (Tozeren et al., 1994; Chao et al., 1996; Shaw et al., 1997; O'Connor et al., 1998) in a phosphoinositide

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3-OH kinase–dependent manner (Shaw et al., 1997). The β4 subunit of this integrin, which contains a cytoplasmic tail of ~1,000 amino acids (Hemler et al., 1989; Kajiji et al., 1989; Kennel et al., 1989), has been shown to be crucial in the ability of this integrin to activate numerous signaling molecules, including phosphoinositide 3-OH kinase (Shaw et al., 1997), Shc (Mainiero et al., 1997), Ras (Mainiero et al., 1997), Jnk (Mainiero et al., 1997), p21WAF1/CIP1 (Clarke et al., 1995), and p53 (Bachelder et al., 1999). The diverse activities of this integrin are exemplified by its ability to promote both the survival of keratinocytes (Dowling et al., 1996) as well as the apoptosis of a number of carcinoma cell lines (Clarke et al., 1995; Kim et al., 1997; Sun et al., 1998; Bachelder et al., 1999). These apparently contradictory functions likely reflect the activation of distinct signaling pathways by this integrin in different cell types as well as the influence of other signaling pathways on α6β4 function.

In the present study, we define opposing signaling pathways that are activated by the α6β4 integrin that promote either carcinoma cell survival or apoptosis, depending on whether these cells express wild-type or functionally inactive mutants of p53. Specifically, we show that α6β4 can promote the AKT/PKB–dependent survival of p53-deficient carcinoma cells. However, this activity contrasts with the ability of α6β4 to stimulate the caspase-dependent cleavage and inactivation of AKT/PKB in p53 wild-type carcinoma cells. The ability of wild-type p53 to inhibit α6β4-associated survival signals suggests that the p53 status of an α6β4-expressing carcinoma cell influences its growth potential.

Materials and Methods

Cells

The RKO colon carcinoma cell line was obtained from M. Brattain (University of Texas, San Antonio, TX), and MDA-MB-435 breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository (Georgetown University).

The cloning of the human β4 cDNA, the construction of the β4 cytoplasmic domain deletion mutant (β4-Δcyt), and their insertions into the pRc/CMV (β4) and pcDNA3 (β4-Δcyt) eukaryotic expression vectors, respectively, have been described (Clarke et al., 1995). RKO/β4Δcyt clone 3E1, RKO/β4 clone D4 (RKO/β4 clone 1), RKO/β4 clone A7 (RKO/β4 clone 2), MDA-MB-435/β4-Δcyt clone 3C12, MDA-MB-435/β4 clone 5B3 (MDA-MB-435/β4 clone 1), and MDA-MB-435/β4 clone 3A7 (MDA/β4 clone 2) were selected for analysis based on their expression of similar surface levels of α6β4 and α6β4-Δcyt, as we have previously demonstrated (Clarke et al., 1995; Shaw et al., 1997; Bachelder et al., 1999).

Dominant negative p53-expressing RKO/β4-Δcyt and RKO/β4 subclones were obtained by cotransfecting RKO/β4-Δcyt clone 3E1 and RKO/β4 clone D4 with plasmids expressing the puromycin resistance gene (Morgenstern and Land, 1990) and a dominant negative p53 (dnp53)[1] construct (provided by M. Oren, Weizmann Institute for Science, Israel) that encodes for a carboxy-terminal domain of p53 that can heterodimerize with endogenous p53 and inhibit its transcriptional activity. Dnp53-expressing subclones were obtained and those subclones expressing high levels of dnp53 were selected by FACS using the Pab122 mAb (Boehringer Mannhein), which recognizes a conserved, denaturation stable epitope in dnp53. In addition, RKO/β4 and RKO/β4-Δcyt cells were transfected with the puromycin resistance gene plasmid alone to obtain puromycin-resistant mock transfectants. All assays were performed using cell maintained below passage 10.

Stable transfectants of MDA/β4 clone 3A7 that expressed temperature-sensitive p53 were obtained by cotransfecting this cell line with plasmids expressing the puromycin resistance gene (1 mg) (Morgenstern and Land, 1990) and a plasmid expressing a temperature-sensitive mutant of human p53 (tsp53; 4 μg) that assumes a functional conformation at 32°C, but not at 37°C (Zhang et al., 1994) using the Lipofectamine reagent (GIBCO BRL). After growing these transfectants in complete medium for 2 d, stable transfectants were selected by culturing these cells in puromycin-containing medium (2 μg/ml) for an additional 18 d. These bulk transfectants were expanded and tsp53 expression was confirmed by showing increased p53 levels in tsp53 transfectants relative to mock transfectants by immunoblotting with a goat anti-human p53, followed by HRP-conjugated donkey anti-goat IgG. All assays were performed on cells maintained below passage 5.

Dominant negative AKT (dnAKT)/PKB–expressing MDA-MB-435/mock and MDA-MB-435/β4 transient transfectants were generated by cotransfecting these cell lines using the Lipofectamine reagent (GIBCO BRL) with a plasmid encoding for green fluorescent protein (pEGFP-1; CLONTECH Laboratories; 1 μg) and a dnAKT/PKB construct that contains inactivating mutations in the catalytic domain of AKT/PKB (4 μg) (Dudek et al., 1997; Skorski et al., 1997; Eves et al., 1998).

Antibodies

The following antibodies were used: 439-9B, a rat mAb specific for the β4 integrin subunit (Falcioni et al., 1998), control rat IgG (Sigma Chemical Co.); Pab122, a polyclonal rabbit serum specific for p53 (Boehringer Mannheim); goat anti-human p53; rabbit polyclonal anti-AKT/PKB raised against a peptide corresponding to mouse AKT/PKB residues 466–479 (New England Biolabs); rabbit polyclonal anti-AKT/PKB phosphoserine 473 (New England Biolabs); rabbit anti-actin (Sigma Chemical Co.); and mouse anti-hemagglutinin (Boehringer Mannheim). Goat anti-mouse IgG and goat anti-rat IgG secondary antibodies, as well as HRP conjugates of these antibodies, were obtained from Jackson ImmunoResearch Laboratories, Inc. HRP-conjugated donkey anti-goat IgG was obtained from BioSource International.

Apoptosis Assays

To induce apoptosis in the RKO and MDA-MB-435 transfectants, the cells were plated in complete medium for 8 h in tissue culture wells (12-well plate; 2.5×10^5 cells/well) that had been coated overnight at 4°C with poly-L-lysine (Sigma Chemical Co.; 2 ml of 25 μg/ml stock) and blocked with 1% BSA. After 8 h, this medium was replaced with serum-free culture medium containing 1% BSA. After 15 h at 37°C, adherent and suspension cells were harvested, combined, and the level of apoptosis in these cells was assessed as described below.

For annexin V stains, cells were washed once with serum-containing medium, once with PBS, once with annexin V-FITC buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), and incubated for 15 min at room temperature with annexin V-FITC (Bender MedSystems) at a final concentration of 2.5 μg/ml in annexin V buffer. After washing once with annexin V buffer, the samples were resuspended in the same buffer and analyzed by flow cytometry. Immediately before analysis, propidium iodide was added to a final concentration of 5 μg/ml to distinguish apoptotic from necrotic cells, and 5,000 cells were analyzed for each sample.

For ApopTag reactions, cells were harvested as described above, fixed in 1% paraformaldehyde for 15 min on ice, and washed twice with PBS. The samples were resuspended in 1 ml ice-cold 70% ethanol and stored at −20°C overnight. After centrifugation at 2,500 rpm for 15 min, cells were washed two times in PBS before performing ApopTag reactions (Oncor) according to the manufacturer's recommendations. These samples were analyzed by flow cytometry.

For in situ analysis of apoptosis in cells transfected transiently with the green fluorescent protein (GFP)–expressing vector pEGFP-1 (CLONTECH Laboratories) and dnAKT/PKB, the transfected cells were stained with annexin V-PE (PharMingen) according to the manufacturer's directions, and plated on coverslips. The percentage of GFP-positive cells that was annexin V-PE–positive was determined by fluorescence microscopy. A total of at least 80 GFP-positive cells from at least 10 microscopic fields were analyzed for each data point.

Analysis of AKT/PKB Expression and Activity

To assess the expression of endogenous AKT/PKB protein, cells were in-

1. *Abbreviations used in this paper:* CAD, caspase-activated deoxyribonuclease; dnAKT, dominant negative AKT; dnp53, dominant negative p53; GFP, green fluorescent protein; HA, hemagglutinin; tsp53, temperature-sensitive p53.

cubated with either rat Ig or 439-9B as described above in the presence of either DMSO (1:500), a caspase 3 inhibitor (Z-DEVD-FMK; Calbiochem-Novabiochem; 4 μg/ml), or a caspase 8 inhibitor (Z-IETD-FMK; Calbiochem-Novabiochem; 4 μg/ml). After washing with PBS, the cells were plated in serum-free medium containing 1% BSA in wells of a 12-well plate that had been coated with anti-rat Ig (13.5 μg/ml) and blocked for 1 h at 37°C with 1% BSA-containing medium. After a 1-h stimulation, adherent and suspension cells were harvested and extracted with AKT/PKB lysis buffer (20 mM Tris, pH 7.4, 0.14 M NaCl, 1% NP-40, 10% glycerol, 2 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 50 μg/ml leupeptin, 1 mM sodium orthovanadate). After removing cellular debris by centrifugation at 12,000 *g* for 10 min, equivalent amounts of total cell protein from these extracts were resolved by SDS-PAGE (8%) and transferred to nitrocellulose. The blots were probed with a rabbit anti-AKT/PKB antiserum, followed by HRP-conjugated goat anti–rabbit Ig, and the immunoreactive bands were visualized by enhanced chemiluminescence. These blots were also probed with a rabbit antiserum specific for actin to confirm the loading of equivalent amounts of protein. Relative AKT/PKB and actin expression levels were assessed by densitometry using IP Lab Spectrum software (Scanalytics).

To determine the level of serine 473–phosphorylated AKT/PKB, cells were transfected transiently using the Lipofectamine reagent (GIBCO BRL) with an HA-tagged AKT/PKB cDNA (provided by A. Toker, Boston Biomedical Research Institute, Boston, MA). 20 h after transfection, these cells were harvested by trypsinization and subjected to antibody-mediated integrin clustering. Specifically, cells were incubated on ice for 30 min with either control rat IgG or 439-9B at a concentration of 10 μg/ml. After washing with PBS, the cells were plated in serum-free medium containing 1% BSA onto wells of a 60-mM tissue culture dish that had been coated at 4°C with anti–rat Ig (13.5 μg/ml) and blocked for 1 h at 37°C in 1% BSA-containing medium. After 1 h, adherent and suspension cells were harvested and washed twice with PBS. Proteins from these cells were extracted with AKT/PKB lysis buffer (see above). After removing cellular debris by centrifugation at 12,000 *g* for 10 min at 4°C, equivalent amounts of total cellular protein were precleared with a 1:1 mixture of protein A and protein G–Sepharose for 1 h at 4°C. Immunoprecipitations were performed for 1 h on these precleared lysates using an HA-specific mAb (1 μg; Boehringer Mannheim) and protein A/protein G–Sepharose beads. Proteins from these immunoprecipitates were subjected to reducing SDS-PAGE (8%), transferred to nitrocellulose, and probed with an AKT/PKB phosphoserine 473–specific rabbit antiserum (New England Biolabs) followed by HRP-conjugated goat anti–rabbit IgG. Phospho-AKT/PKB was detected on these blots by chemiluminescence (Pierce Chemical Co.). These samples were also probed with rabbit anti-AKT/PKB. The relative intensity of phosphoserine AKT/PKB and AKT/PKB bands was assessed by densitometry, as described above.

Analysis of AKT/PKB Proteolysis

Baculovirus-expressed AKT/PKB (0.5 μg; provided by A. Toker) was incubated with either active recombinant caspase 8 (2 mg; Calbiochem-Novabiochem) or active recombinant caspase 3 (2 μg; Calbiochem-Novabiochem) at 37°C for 1 h in a final volume of 10 μl. Subsequently, the reaction mixtures were divided into two aliquots and resolved by SDS-PAGE (8%). The gels were silver stained using the GelCode SilverSNAP Stain Kit (Pierce Chemical Co.) or transferred to nitrocellulose and probed with a rabbit AKT/PKB antiserum as described above.

Results

The α6β4 Integrin Promotes the Survival of p53-deficient, but Not p53 Wild-type Carcinoma Cells

For our initial experiments, we used stable β4 transfectants of two α6β4-deficient carcinoma cell lines that differ in their p53 status: RKO colon carcinoma cells, which express wild-type p53 (Nagasawa et al., 1995); and MDA-MB-435 breast carcinoma cells, which express a mutant, inactive form of p53 (Lesoon-Wood et al., 1995). We also used RKO and MDA-MB-435 cells that express a cytoplasmic domain deletion mutant of α6β4 (RKO/β4-Δcyt; MDA/b4-Δcyt) that is signaling deficient. The characterization of these cells has been described previously (Clarke et al., 1995; Shaw et al., 1997).

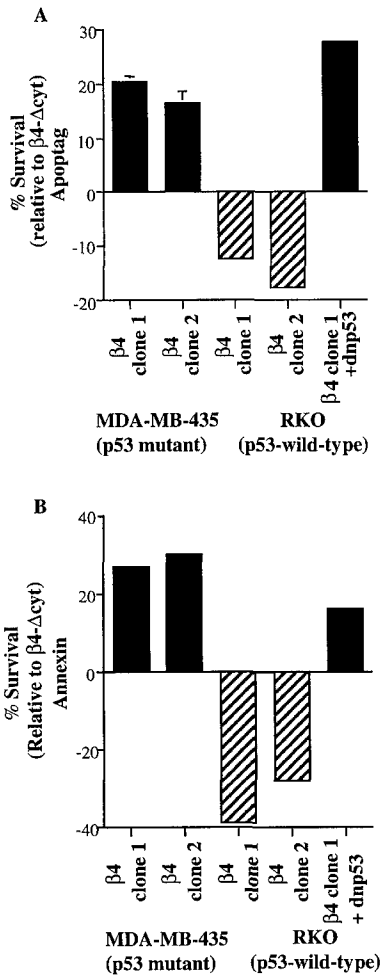

Figure 1. p53 inhibits α6β4-mediated survival. MDA-MB-435, RKO, and RKO + dnp53 cells that expressed either α6β4 (β4) or α6β4-Δcyt (β4-Δcyt) were plated on poly-L-lysine–coated tissue culture wells and cultured in the absence of serum. After 15 h, the cells were harvested, subjected to either ApopTag reactions (A) or annexin V-FITC staining (B), and analyzed by flow cytometry. A survival effect of α6β4 was quantified by subtracting the percentage of α6β4-expressing cells that were positive for either Apoptag (A) or annexin V-FITC (B) staining from the percentage of α6β4-Δcyt–expressing cells that were positive for these markers. This value was plotted on the bar graphs shown in A and B, with positive values indicating that the specified β4 clone exhibits increased survival relative to the relevant β4-Δcyt subclone, and negative values indicating an increased apoptosis of the indicated clone relative to the appropriate β4-Δcyt clone. The data in A represent the means (± SEM) from three independent experiments. Similar results to those shown in B were observed in three separate trials.

To explore the potential influence of α6β4 expression on the survival of serum-starved carcinoma cells deprived of matrix attachment, the α6β4 and α6β4-Δcyt–expressing RKO and MDA-MB-435 subclones were plated on poly-L-lysine in serum-free medium. The level of apoptosis in these populations was determined either by staining with annexin V-FITC to detect cells in the early stages of apoptosis or by performing terminal deoxynucleotidyl trans-

Table I. Influence of α6β4 Integrin on the Viability of RKO and MDA-MB-435 Cells

Clone	Percent propidium iodide–positive cells
MDA/Mock	21
β4 Clone 1	13
β4 Clone 2	9
RKO/Mock	32
β4 Clone 1	49
β4 Clone 2	47

Mock-transfected and β4-transfected MDA-MB-435 and RKO cells were plated on poly-L-lysine (25 μg/mL) in the absence of serum for 15 h, harvested, and incubated with propidium iodide (PI). The percentage of PI-positive cells was assessed by flow cytometry. Similar results were observed in four independent experiments.

ferase end labeling reactions (Apoptag) to detect DNA fragmentation (Fig. 1). In addition, we assessed the viability of these serum-deprived cells by measuring the cellular uptake of propidium iodide (Table I). The ability of α6β4 to promote the survival of these cells was determined by subtracting the percent apoptotic α6β4-expressing cells from the percent apoptotic α6β4-Δcyt–expressing cells. The expression of α6β4 in MDA-MB-435 cells significantly increased the survival of these cells relative to MDA-MB-435 cells expressing α6β4-Δcyt, as assessed by annexin V-FITC staining (Fig. 1), ApopTag staining (Fig. 1), and propidium iodide uptake (Table I). In contrast, the expression of α6β4 in RKO cells did not increase the survival of these cells relative to either the mock (Table I) or RKO/β4-Δcyt transfectants (Fig. 1). In fact, we observed a higher level of apoptosis and cell death in serum-starved RKO/β4 as compared with RKO/β4-Δcyt cells, in agreement with our previous demonstration that α6β4 can promote apoptosis in wild-type p53 carcinoma cells (Bachelder et al., 1999).

Based on the fact that RKO and MDA-MB-435 cells differ in their p53 status, we reasoned that the ability of α6β4 to promote cell survival may be inhibited by p53. This hypothesis was examined by investigating the effect of α6β4 expression on the survival of RKO cells in which p53 activity had been inhibited by the expression of a dnp53 construct. Indeed, α6β4 expression promoted the survival of serum-starved, dnp53-expressing RKO cells as determined by ApopTag and annexin V-FITC staining (Fig. 1). These results demonstrate that p53 can suppress the survival signaling mediated by α6β4 in serum-starved carcinoma cells.

α6β4-Mediated Survival in p53-deficient Carcinoma Cells Is Inhibited by Dominant Negative AKT/PKB

Given the importance of the AKT/PKB kinase in numerous survival signaling pathways (Ahmed et al., 1997; Datta et al., 1997; Dudek et al., 1997; Songyang et al., 1997; Blume-Jensen et al., 1998; Crowder and Freeman, 1998; Gerber et al., 1998), we investigated whether the survival function of α6β4 in serum-starved, p53-deficient carcinoma cells was AKT/PKB–dependent. The MDA-MB-435/β4–transfected clones, as well as the parental cells, were cotransfected with plasmids encoding for GFP and an HA-tagged, kinase-deficient AKT/PKB mutant that acts as a dominant negative construct (dnAKT/PKB) (Dudek et al., 1997; Skorski et al., 1997; Eves et al., 1998). Expression of this dnAKT/PKB construct was confirmed by immunoblotting extracts from these transfected cells with an HA-specific mAb (data not shown). After 15 h of serum starvation, the level of apoptosis in GFP-positive cells was assessed by annexin V-PE staining. As shown in Fig. 2, MDA-MB-435/β4 clones demonstrated significantly less apoptosis than parental MDA-MB-435 cells in agreement with the data shown in Table I. Importantly, dnAKT/PKB expression inhibited this α6β4 survival function in each of the two MDA-MB-435/β4 clones examined, but it did not alter the level of apoptosis in parental MDA-MB-435 cells.

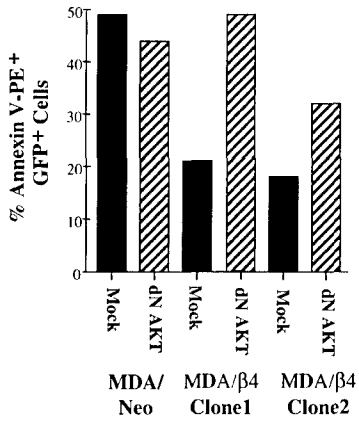

Figure 2. Expression of a dominant negative AKT/PKB inhibits α6β4-mediated survival. Parental (neo) and α6β4-expressing (β4) MDA-MB-435 cells were transfected with either a GFP-expressing plasmid (mock) or both a GFP and a dnAKT/PKB–expressing construct (dnAKT/PKB), plated on poly-L-lysine, and cultured for 15 h in the absence of serum. Apoptosis in these cells was assessed by annexin V-PE staining. The data are reported as the percentage of GFP-positive cells that were stained by annexin V-PE. Similar results were observed in two additional experiments.

p53 Inhibits the Activation of AKT/PKB by α6β4

To understand the mechanism by which p53 inhibits α6β4-mediated survival, we investigated the possibility that p53 alters the ability of this integrin to activate AKT/PKB. Initially, we examined whether the antibody-mediated clustering of α6β4 in MDA-MB-435 cells resulted in the phosphorylation of AKT/PKB on serine 473, an event that has been shown to correlate with AKT/PKB activation (Alessi et al., 1996). MDA-MB-435/β4 subclones were transfected with an HA-tagged AKT/PKB construct. These cells were incubated with either a control rat IgG or the β4-specific antibody 439-9B and plated in the absence of serum on secondary antibody–coated tissue culture wells for 1 h. HA immunoprecipitations were performed on extracts from these cells, and the levels of serine-phosphorylated AKT/PKB were assessed by blotting these immunoprecipitates with an antiserum specific for AKT/PKB molecules phosphorylated on serine residue 473. As shown in Fig. 3 A, the antibody-mediated clustering of α6β4 stimulated an increase in the level of serine-phosphorylated AKT/PKB in each of the two MDA-MB-435/β4 subclones relative to control cells (2.1-fold increase, β4 clone 1; 5.5-fold increase, β4 clone 2). This α6β4-induced increase in AKT/

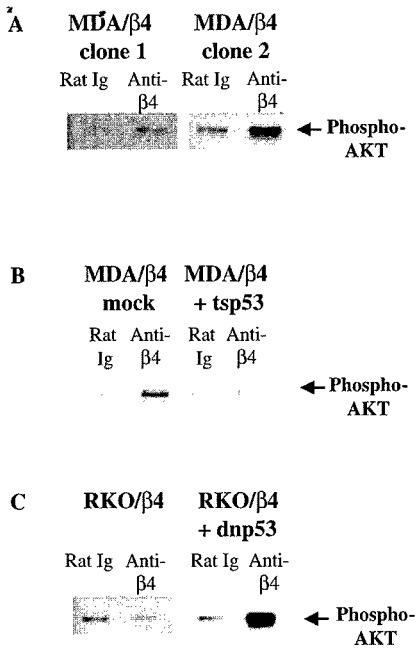

Figure 3. p53 inhibits the ability of α6β4 to induce AKT/PKB phosphorylation in carcinoma cells. MDA/β4, MDA/β4 + tsp53, RKO/β4, and RKO/β4 + dnp53 cells were transfected transiently with an HA-tagged AKT/PKB. These transfectants were incubated with the indicated primary antibodies, washed, and plated in the absence of serum on secondary antibody–coated tissue culture wells. HA-AKT/PKB–transfected MDA/β4 (A), RKO/β4 (C), and RKO/β4 + dnp53 (C) cells were stimulated for 1 h at 37°C. Alternatively, mock- and tsp53-transfected MDA/β4 cells (B) were stimulated for 1 h at 32°C to activate tsp53, followed by an additional hour at 37°C to activate AKT/PKB. Immunoprecipitations were performed with an HA-specific mAb on equal amounts of total extracted protein. The immunoprecipitates were resolved by SDS-PAGE (8%), transferred to nitrocellulose, and probed with a phosphoserine 473 AKT/PKB–specific rabbit antiserum (New England Biolabs), followed by HRP-conjugated goat anti–rabbit IgG. Phosphoserine-specific AKT/PKB bands were detected by chemiluminescence, and are noted by arrows.

PKB serine phosphorylation was dependent on α6β4 signaling based on the inability of α6β4-Δcyt clustering to increase the level of the serine 473–phosphorylated AKT/PKB in MDA-MB-435/β4-Δcyt subclones (data not shown).

To investigate the influence of p53 on the activation of AKT/PKB by α6β4, we explored whether α6β4 clustering induced the phosphorylation of AKT/PKB on serine residue 473 in MDA-MB-435/β4 that had been reconstituted with functional p53. Specifically, MDA-MB-435/β4 cells were transfected with a temperature-sensitive mutant of human p53 (tsp53) that assumes a functional conformation at 32°C but not at 37°C (Zhang et al., 1994). This construct has been used extensively to study the influence of p53 on signaling pathways involved in cell growth and apoptosis (Kobayashi et al., 1995; Owen-Schaub et al., 1995). Stable transfectants of these cells were selected, and tsp53 expression was confirmed by immunoblotting (data not shown). Tsp53 and mock-transfected cells were transfected transiently with HA-AKT/PKB. After incubating these cells with either rat IgG or 439-9B, they were plated on secondary antibody–coated wells and subjected to a 32°C incubation to stimulate p53 activity, followed by a 37°C incubation to activate AKT/PKB. HA immunoprecipitations were performed on extracts from these cells, and these immunoprecipitates were subjected to immunoblotting with phosphoserine 473 AKT/PKB–specific rabbit antiserum. As shown in Fig. 3 B, the clustering of α6β4 significantly increased the level of phosphoserine 473-AKT/PKB in mock-transfected MDA/β4 cells (7.9-fold increase), but not in tsp53-expressing MDA/β4 cells (1.2-fold increase). The importance of p53 in the inhibition of the α6β4-associated activation of AKT/PKB was indicated by the finding that α6β4 clustering increased the level of phosphoserine 473 AKT/PKB in MDA/β4 + tsp53 transfectants that had been incubated at 37°C, the nonpermissive temperature for this tsp53 construct (data not shown).

The ability of p53 to suppress the α6β4-mediated activation of AKT/PKB was explored further in RKO carcinoma cells, which express wild-type p53. In agreement with the results obtained in MDA/β4 cells that had been reconstituted with functional p53, the clustering of α6β4 in two independent RKO/β4 subclones did not result in increased amounts of serine phosphorylated AKT/PKB (Fig. 3 C and data not shown). Importantly, the expression of dnp53 in RKO/β4 cells restored the ability of α6β4 to activate AKT/PKB, as evidenced by an increase in phosphoserine 473-AKT/PKB immunoreactivity in RKO/β4 + dnp53 cells that had been subjected to antibody-mediated α6β4 clustering (8.6-fold increase), as described above (Fig. 3 C). The ability of α6β4 to stimulate AKT/PKB activity in RKO/β4 + dnp53 cells but not in RKO/β4 cells was confirmed by performing in vitro kinase assays using histone H2B as a substrate (data not shown). As a control for specificity, we also demonstrated that the clustering of α6β4 on dnp53-expressing RKO/β4-Δcyt cells did not stimulate AKT/PKB activity (data not shown).

α6β4 Stimulation Induces the Caspase 3–dependent Cleavage of AKT/PKB in a p53-dependent Manner

To define the mechanism by which p53 inhibits the ability of α6β4 to activate AKT/PKB, we investigated whether p53 alters AKT/PKB expression levels in response to α6β4 clustering. RKO/β4 and RKO/β4 + dnp53-expressing cells were incubated with either rat Ig or 439-9B and stimulated on secondary antibody–coated wells for 1 h. The amount of total AKT/PKB in equivalent amounts of total protein from these lysates was assessed by immunoblotting. Importantly, the antibody-mediated clustering of the α6β4 integrin on each of two RKO/β4 subclones resulted in a significant reduction in the total level of AKT/PKB in these cells (Fig. 4 A). In contrast, AKT/PKB levels were not reduced in dnp53-expressing RKO/β4 cells (Fig. 4 B) or in MDA-MB-435/β4 subclones (data not shown) after the antibody-mediated clustering of α6β4. We also observed decreased levels of HA-AKT/PKB protein in HA-AKT/PKB–transfected RKO/β4 cells, but not in HA-AKT/PKB–transfected RKO/β4 + dnp53 cells upon the antibody-mediated clustering of α6β4 (data not shown).

Based on the reported ability of caspases to cleave signaling molecules that promote cell survival (Cheng et al.,

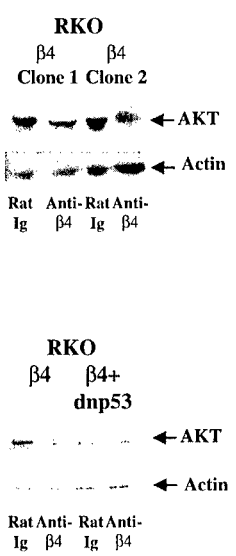

Figure 4. Clustering of the α6β4 integrin reduces AKT/PKB protein levels in p53-wild type but not in p53-deficient carcinoma cells. RKO/β4 (A and B) and RKO/β4 + dnp53 (B)–expressing cells were incubated with either rat Ig or 439-9B and plated on secondary antibody–coated wells for 1 h in the absence of serum. Equivalent amounts of total protein from lysates from these cells were resolved by SDS-PAGE (8%), transferred to nitrocellulose, and probed with an AKT/PKB–specific rabbit antiserum (New England Biolabs) followed by HRP-conjugated goat anti–rabbit IgG. These blots were also probed with an actin-specific rabbit antiserum (Sigma Chemical Co.) to confirm the loading of equivalent amounts of protein. The AKT/PKB and actin bands were detected by enhanced chemiluminescence, and are indicated by arrows. These bands were quantified by densitometry. α6β4 clustering decreased AKT/PKB levels in RKO/β4 subclones (1.7-fold decrease, β4 clone 1; 1.9-fold decrease, β4 clone 2), but not in RKO/β4 + dnp53 cells. Similar results were observed in four additional trials.

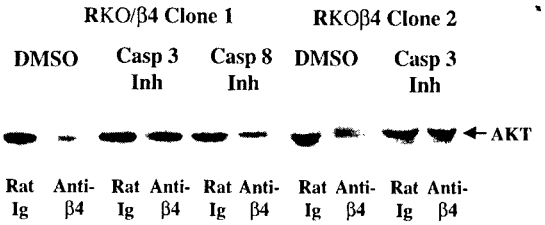

Figure 5. A caspase 3 inhibitor blocks α6β4-associated reductions in AKT/PKB protein levels. RKO/β4 cells were incubated with either rat Ig or 439-9B in the presence of DMSO (1:500), a caspase 3 inhibitor (Z-DEVD-FMK; 4 μg/ml), or a caspase 8 inhibitor (Z-IETD-FMK; 4 μg/ml). These cells were washed with PBS and plated onto secondary antibody–coated wells in the presence of the same drugs for 1 h in serum-free medium. Equivalent amounts of total protein were resolved by SDS-PAGE (8%), transferred to nitrocellulose, and probed with an AKT/PKB–specific rabbit antiserum (New England Biolabs) followed by HRP-conjugated goat anti–rabbit IgG. AKT/PKB was detected by enhanced chemiluminescence and quantified by densitometry. The antibody-mediated clustering of α6β4 decreased the level of AKT/PKB in DMSO-treated cells (2.0-fold decrease, β4 clone 1; 1.9-fold decrease, β4 clone 2), as well as in cells pretreated with a caspase 8 inhibitor (1.9-fold decrease). In contrast, the pretreatment of these cells with a caspase 3 inhibitor partially restored AKT/PKB levels in RKO/β4 cells subjected to α6β4 clustering (1.1-fold decrease, β4 clone 1; 1.1-fold decrease, β4 clone 2). By probing these blots with an actin-specific rabbit antiserum (Sigma Chemical Co.), we confirmed that equivalent amounts of actin were present in each lane (data not shown). Similar results were observed in three experiments.

1997; Enari et al., 1998; Sakahira et al., 1998), we hypothesized that α6β4 may promote the caspase-dependent cleavage of AKT/PKB in wild-type p53-expressing carcinoma cells. Initially, we explored the importance of caspase 3 activity, which has been shown to play a crucial role in p53-dependent apoptotic pathways (Fuchs et al., 1997), in the α6β4-associated reduction of AKT/PKB expression levels. In agreement with the data shown in Fig. 4, the clustering of α6β4 in control RKO/β4 cells significantly reduced the level of AKT/PKB in these carcinoma cells (Fig. 5). However, RKO/β4 cells that had been pretreated with Z-DEVD-FMK, a cell permeable caspase 3 inhibitor, did not exhibit decreased levels of AKT/PKB in response to α6β4 clustering (Fig. 5). In contrast, we detected a decreased amount of AKT/PKB after the clustering of α6β4 in RKO/β4 cells that had been pretreated with Z-IETD-FMK, a cell permeable caspase 8 inhibitor (Fig. 5). Importantly, no effect of these inhibitors on AKT/PKB levels was observed upon the clustering of α6β4 on RKO/α6β4-Δcyt cells (data not shown).

The ability of the caspase 3 inhibitor to restore normal AKT/PKB levels suggested that AKT/PKB is cleaved by caspase 3 upon the clustering of α6β4 in carcinoma cells expressing wild-type p53. To establish the caspase 3–mediated cleavage of AKT/PKB more rigorously, we investigated whether a recombinant form of this cysteine protease could cleave baculovirus-expressed AKT/PKB in vitro. Proteins in these reactions were resolved by SDS-PAGE and detected by silver staining. The results obtained revealed that the incubation of baculovirus-expressed AKT/PKB (M_r, 60 kD) with recombinant caspase 3 resulted in the formation of an AKT/PKB cleavage product (M_r, 49 kD) (Fig. 6). In contrast, we did not detect an AKT/PKB cleavage product after the incubation of baculovirus AKT/PKB with recombinant caspase 8 (Fig. 6). The caspase 3–generated AKT/PKB cleavage product was also detected by immunoblotting with an antiserum specific for the carboxy terminus of AKT/PKB, suggesting that caspase 3 cleaves AKT/PKB at its amino terminus (data not shown).

Finally, to demonstrate that the caspase 3–dependent cleavage of AKT/PKB was responsible for the p53 inhibition of AKT/PKB activity in RKO/β4 cells, we explored the effects of a caspase 3 inhibitor on the ability of α6β4 to activate AKT/PKB. HA-AKT/PKB–transfected RKO/β4 cells were subjected to antibody-mediated α6β4 clustering in the presence of either DMSO or the caspase 3 inhibitor Z-DEVD-FMK. HA immunoprecipitates from extracts from these cells were subjected to immunoblotting with the phosphoserine 473 AKT/PKB–specific rabbit antiserum. As shown in Fig. 7, the pretreatment of RKO/β4 cells with Z-DEVD-FMK restored the ability of α6β4 to stimulate the phosphorylation of AKT/PKB in these cells. These results demonstrate that α6β4 stimulates the caspase 3–dependent cleavage and inactivation of AKT/PKB in p53 wild-type, but not in p53-deficient carcinoma cells.

Discussion

The binding of extracellular matrix proteins to integrins initiates survival signals that inhibit anoikis, a form of apoptosis induced upon the detachment of cells from extracellular matrix (Meredith et al., 1993; Frisch and Francis,

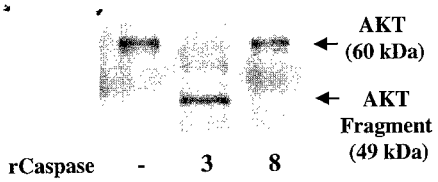

Figure 6. AKT/PKB is cleaved by recombinant caspase 3 in vitro. Baculovirus-expressed AKT/PKB (0.5 μg) was incubated either alone, with recombinant caspase 3 (2 μg) or with recombinant caspase 8 (2 μg) for 1 h at 37°C. Proteins in these reactions were resolved by SDS-PAGE (8%) and subjected to silver staining. AKT/PKB and its cleavage product are indicated by arrows. Similar results were observed in three trials.

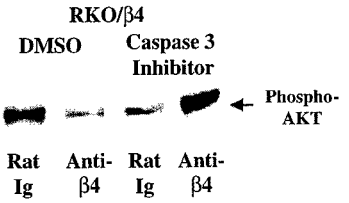

Figure 7. A caspase 3 inhibitor restores the ability of α6β4 to induce AKT/PKB phosphorylation. HA-AKT/PKB–transfected RKO/β4 cells were incubated with either rat Ig or 439-9B in the presence of DMSO (1:500) or a caspase 3 inhibitor (Z-DEVD-FMK; 4 μg/ml). After washing with PBS, these cells were plated on secondary antibody–coated wells in serum-free medium containing the indicated drugs for 1 h. HA immunoprecipitations were performed on equivalent amounts of total extracted protein from these samples. These immunoprecipitates were resolved by SDS-PAGE (8%), transferred to nitrocellulose, and probed with rabbit antiserum specific for phosphoserine 473-AKT/PKB, followed by HRP-conjugated goat anti–rabbit Ig. Phosphoserine 473-AKT/PKB was detected by enhanced chemiluminescence, and is indicated by an arrow. Total AKT/PKB levels were also assessed by stripping these membranes and probing with an AKT/PKB–specific rabbit antiserum (data not shown). Relative activity was assessed by determining the ratio of serine phosphorylated AKT/PKB to that of total AKT/PKB for each sample (relative AKT activity: lane 1 = 1.0; lane 2 = 1.3; lane 3 = 1.1; and lane 4 = 3.1). Similar results were observed in three experiments.

1994). In the current studies, we show that the α6β4 integrin suppresses anoikis exclusively in carcinoma cells that lack functional p53. Furthermore, we demonstrate that this α6β4-associated survival function depends on the ability of this integrin to activate the serine/threonine kinase AKT/PKB in p53-deficient cells. Finally, we provide evidence that p53 inhibits the α6β4-mediated activation of AKT/PKB by promoting the caspase 3–dependent cleavage of this kinase. Collectively, our findings establish that p53 can inhibit an integrin-associated survival function, a phenomenon that has important implications for tumor cell growth.

Our results suggest that the α6β4 integrin can enhance the survival of carcinoma cells in an AKT/PKB–dependent manner. Although previous studies have shown that cell attachment to matrix proteins promotes the survival of primary epithelial cells (Khwaja et al., 1997; Farrelly et al., 1999), α6β4 is the first specific integrin to be implicated in the delivery of AKT/PKB–dependent survival signals to carcinoma cells. The importance of AKT/PKB in α6β4 survival signaling was indicated in our studies by the ability of a dnAKT/PKB construct containing inactivating mutations in the catalytic domain to inhibit the survival effect of α6β4 in serum-starved MDA-MB-435 cells. Although this dnAKT/PKB has been used extensively to implicate AKT/PKB in survival pathways, it is possible that it associates with phosphoinositide-dependent kinases and inhibits their activity. However, our observation that the expression of a constitutively active AKT/PKB in MDA-MBA-435 enhances their survival (data not shown) strongly suggests that α6β4 expression promotes the survival of these cells by activating AKT/PKB.

Our demonstration that p53 can inhibit AKT/PKB kinase activity is of interest in light of the recent finding that the PTEN tumor suppressor can also inhibit cell growth by inhibiting AKT/PKB in a manner that is dependent on its lipid phosphatase activity (Myers et al., 1998; Stambolic et al., 1998; Davies et al., 1999; Ramaswamy et al., 1999; Sun et al., 1999). Together, our current findings on p53 and the previously described activities of PTEN highlight the impact of tumor suppressors on integrin-mediated functions. Moreover, our demonstration that p53 inhibits α6β4 survival signaling by promoting the caspase-dependent cleavage of AKT/PKB provides a mechanistic link between tumor suppressor function and the regulation of integrin signaling, similar to the phosphatase activities of PTEN. Although previous studies have demonstrated that caspases can be activated by p53 in both cell-free systems (Ding et al., 1998) as well as in response to DNA damage (Fuchs et al., 1997; Yu and Little, 1998), our findings suggest that caspases can also be activated by an integrin in a p53-dependent manner. Indeed, it will be informative to determine if other activators of p53 such as DNA damage (Siegel et al., 1995; Komarova et al., 1997) can promote the caspase-dependent cleavage of AKT/PKB.

The finding that AKT/PKB activity can be regulated by caspase 3 substantiates the hypothesis that caspases play an important role in many forms of apoptosis based on their ability to cleave signaling molecules that influence cell survival. For example, caspases have been shown to cleave and inactivate an inhibitor of caspase-activated deoxyribonuclease (CAD). Importantly, the cleavage of this inhibitor results in the activation of CAD, which is the enzyme responsible for the DNA fragmentation that is characteristic of apoptosis (Enari et al., 1998; Sakahira et al., 1998). Caspase 3 has also been shown to cleave bcl-2, resulting in an inhibition of its anti-apoptotic function (Cheng et al., 1997). While AKT/PKB has been suggested to be a target of caspase activity based on the reduced levels of this kinase observed in T cells in response to fas stimulation (Widmann et al., 1998), our results extend this finding by establishing definitively that AKT/PKB is cleaved by caspase 3. More importantly, we provide evidence that this cleavage event results in the inhibition of AKT/PKB kinase activity, and implicate this event in the inhibition of α6β4 integrin survival function.

It is important to consider the mechanism by which the α6β4-induced, caspase-dependent cleavage of AKT/PKB inhibits its kinase activity. We detected an AKT/PKB fragment (M_r, 49 kD) after the in vitro incubation of AKT/

PKB with recombinant caspase 3. This fragment was recognized by a rabbit antiserum raised against a peptide corresponding to the extreme carboxy-terminal amino acids of the molecule, suggesting that caspase 3 cleaves AKT/PKB at its amino terminus. Interestingly, the pleckstrin homology domain, which resides in the amino terminus of AKT/PKB, is important in both the translocation of this kinase to the membrane and its subsequent activation (Franke et al., 1995; Andjelkovic et al., 1997). It is possible that the caspase 3–dependent cleavage of AKT/PKB prevents the membrane translocation of this kinase, thus, preventing its activation. However, we were unable to identify an AKT/PKB fragment in vivo after the clustering of α6β4, despite our detection of reduced AKT/PKB levels under these conditions. This result suggests that after the initial cleavage of AKT/PKB by caspase 3, this kinase is subjected to further cleavage by other caspases, as has been shown for ICAD (Tang and Kidd, 1998). Moreover, our inability to detect AKT/PKB fragments in vivo after the clustering of α6β4 suggests that AKT/PKB cannot be detected by immunoblotting after its cleavage by multiple caspases. The ability of a caspase 3 inhibitor to restore both normal AKT/PKB levels as well as the α6β4-mediated activation of AKT/PKB suggests that the degradation of AKT/PKB observed in vivo is dependent on the initial cleavage of this kinase by caspase 3.

In contrast to our finding that p53-dependent, caspase 3 activity inhibits AKT/PKB, other studies have concluded that constitutively active AKT/PKB can delay p53-dependent apoptosis (Sabbatini and McCormick, 1999), inhibit caspases (Cardone et al., 1998), and block caspase-dependent forms of apoptosis (Berra et al., 1998; Gibson et al., 1999). The demonstrated ability of AKT/PKB to inhibit p53 and caspase activity in these studies may relate to the kinetics of AKT/PKB activation. Specifically, the rapid stimulation of AKT/PKB may impede p53 or caspase activation. In contrast, the ability of α6β4 clustering to promote the caspase 3–dependent inactivation of AKT/PKB in p53 wild-type carcinoma cells may relate to the fact that α6β4 signaling stimulates caspase activity before AKT/PKB activity in these cells. Alternatively, it is possible that the ability of caspase 3 to cleave AKT/PKB was not observed in previous studies because insufficient amounts of endogenous caspase activity were present to inhibit the activity of exogenously introduced, active AKT/PKB. Nonetheless, these results suggest that an intimate crosstalk exists between AKT/PKB and caspases that contributes to the regulation of cell survival.

We have previously demonstrated that the α6β4 integrin activates p53 function (Bachelder et al., 1999). The current studies describe an important consequence of this α6β4 activity, namely the inhibition of AKT/PKB activity and its associated cell survival function. Similar to previous results from our laboratory (Clarke et al., 1995; Shaw et al., 1997; O'Connor et al., 1998) and others (Kim et al., 1997; Sun et al., 1998), the current studies demonstrate that the survival function of α6β4 is ligand-independent in β4-transfected, p53-deficient carcinoma cells. This ligand-independent survival function may be attributable to the ability of the β4 cytoplasmic domain to self-associate (Rezniczek et al., 1998).

In addition to demonstrating that p53 inhibits α6β4-mediated survival, we observed that α6β4 increases the level of apoptosis observed in serum-starved p53 wild-type carcinoma cells. This result suggests that the apoptotic signaling pathway activated by α6β4 can augment the apoptotic signaling initiated by serum deprivation. Although p53 has been implicated in the apoptosis induced in endothelial cells upon their detachment from matrix (Ilic et al., 1998), others have reported that epithelial cell anoikis is p53-independent (Boudreau et al., 1995). In agreement with the results of the latter study, we observed apoptosis in p53-deficient cells, including MDA-MB-435 cells and dnp53-expressing RKO cells, upon their detachment from matrix. These results indicate that carcinoma cells are subject to a p53-independent form of anoikis. In combination with our previous observation that α6β4 apoptotic signaling requires p53 activity (Bachelder et al., 1999), our findings suggest that the p53-independent apoptosis of carcinoma cells that occurs in response to matrix detachment can be enhanced by p53-dependent, α6β4 apoptotic signaling.

The current studies may explain why the α6β4 integrin has been implicated in the apoptosis of some cells and the survival of others. Specifically, α6β4 has been shown to induce growth arrest and apoptosis in several carcinoma cell lines (Clarke et al., 1995; Kim et al., 1997, Sun et al., 1998) as well as in endothelial cells (Miao et al., 1997). However, this integrin has also been shown to promote the proliferation (Mainiero et al., 1997; Murgia et al., 1998) and survival (Dowling et al., 1996) of keratinocytes. These apparently contradictory functions of α6β4 may relate to the fact that the functions of α6β4 are cell type–specific. The current studies establish that the p53 tumor suppressor is one critical signaling molecule that may influence α6β4 function in different cell types because this integrin promotes apoptosis only in wild-type p53-expressing cells and survival only in p53-deficient cells. Interestingly, the reported ability of α6β4 to promote keratinocyte survival (Dowling et al., 1996) may relate to the reported deficiency of p53 activity in these cells (Nigro et al., 1997).

One implication of our findings is that the α6β4 integrin is similar to a number of oncogenes that promote cell proliferation in some settings and cell death in others. The recent observation that oncogenes can deliver such death signals has led to their seemingly contradictory categorization as tumor suppressors in select environments. For example, although the stimulation of c-myc and E2F normally promotes cell proliferation, the activation of these oncogenes induces apoptosis in the presence of secondary stress signals such as p53 expression, serum starvation or hypoxia (Evan et al., 1992; Shi et al., 1992, Hermeking and Eick, 1994; Qin et al., 1994; Wu and Levine, 1994). The ability of these stress signals to stimulate oncogene-dependent apoptosis is thought to be important in eliminating tumor cells that escape normal proliferation checkpoints as a result of oncogene expression. Similarly, the α6β4 integrin, which promotes the survival of p53-deficient cells, could also be classified loosely as a tumor suppressor based on its apoptotic function in carcinoma cells that express wild-type p53. The current studies demonstrate that, similar to the activity of oncogenes, integrin function and signaling can be profoundly influenced by physiological stimuli that activate other signaling pathways in a cell.

In summary, we have described the ability of the α6β4

integrin to promote the survival of the p53 mutant, but not p53 wild-type carcinoma cells. This ability of p53 to influence integrin-mediated functions so markedly derives from its ability to activate the caspase 3–dependent cleavage of AKT/PKB. The fact that AKT/PKB overexpression has been suggested to contribute to the transformed phenotype of tumor cells (Bellacosa et al., 1995) suggests that the introduction of the α6β4 integrin into p53 wild-type tumors may inhibit their growth by inducing the cleavage of this transforming protein. The ability of α6β4 to induce the p53-dependent cleavage of AKT/PKB also suggests that the acquisition of inactivating mutations in either p53 or caspase 3 will provide a selective growth advantage for carcinoma cells by stimulating α6β4-mediated AKT/PKB–dependent survival signaling. Moreover, given our previous demonstration that α6β4 promotes carcinoma cell migration and invasion (Chao et al., 1996, Shaw et al., 1997; O'Connor et al., 1998), we suggest that carcinoma cells that express α6β4 and mutant forms of p53 or caspase 3 will have a distinct advantage in their ability to disseminate and survive as metastatic lesions.

We thank Moshe Oren, Alt Zantema, Alex Toker, and Phil Hinds (Harvard Medical School, Boston, MA) for reagents. We also thank Lewis Cantley, Alex Toker, Phil Hinds, Kathy O'Connor, and Leslie Shaw (Beth Israel Deaconess Medical Center, Boston, MA) for valuable discussions.

This work was supported by National Institutes of Health grants CA80789, AI39264 (both to A.M. Mercurio), and CA81697 (to R.E. Bachelder), as well as by the Italian Association for Cancer Research.

Submitted: 14 May 1999
Revised: 13 October 1999
Accepted: 18 October 1999

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 14, Issue of April 7, pp. 10604–10610, 2000
Printed in U.S.A.
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Cooperative Signaling between $\alpha_6\beta_4$ Integrin and ErbB-2 Receptor Is Required to Promote Phosphatidylinositol 3-Kinase-dependent Invasion*

(Received for publication, June 10, 1999, and in revised form, November 19, 1999)

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We previously demonstrated that β_4 integrin subunit overexpression increases *in vitro* invasiveness of NIH3T3 cells that have been transformed by ErbB-2 oncogene. We used this model to identify domains within the large β_4 cytoplasmic domain that are involved in the interaction of $\alpha_6\beta_4$ with ErbB-2, invasion, and phosphatidylinositol 3-kinase (PI3K) activation. For this purpose, we expressed deletion mutants of β_4 that lacked either all or portions of the β_4 cytoplasmic domain in NIH3T3/ErbB-2 cells. We also used an ecto-domain mutant in which most of the extracellular domain of β_4 was replaced with a c-Myc tag. These transfectants were examined for their ability to invade Matrigel and their ability to activate PI3K, as well as for the ability of $\alpha_6\beta_4$ to co-immunoprecipitate with ErbB-2. The results obtained revealed that a region of the β_4 cytoplasmic domain between amino acids 854 and 1183 is critical for the ability of $\alpha_6\beta_4$ integrin to increase invasion. Interestingly, the extracellular domain of β_4 is not necessary for $\alpha_6\beta_4$ to stimulate invasion. The association of $\alpha_6\beta_4$ with ErbB-2 is dependent upon the β_4 cytoplasmic domain and can occur in the absence of $\alpha_6\beta_4$ heterodimerization. Finally, we observed strong activation of PI3K with β_4 wild type and with those β_4 deletion mutants that were able to stimulate invasion upon the expression in NIH3T3/ErbB-2 cells. In conclusion, our results establish that there is cooperation between $\alpha_6\beta_4$ and ErbB-2 in promoting PI3K-dependent invasion and implicate a specific region of the β_4 cytoplasmic domain (amino acids 854–1183) in this event.

Integrins are the major family of cell surface receptors that mediate attachment to the extracellular matrix. The interaction between integrins and their ligands is involved in the regulation of many cellular functions, including embryonic development, cell proliferation, as well as tumor growth and metastasis. Integrins are composed of α and β transmembrane subunits that heterodimerize to form different receptors. A single α subunit (*e.g.* α_v or α_6) can associate with different β subunits, (β_1, β_3, β_5, or β_1, β_4, respectively), promoting different ligand binding specificity (1–3). The $\alpha_6\beta_4$ integrin is a receptor for various isoforms of the basement membrane component laminin (4–6), and its expression is restricted to epithelia, endothelia and peripheral nerves (7–9). In many epithelia, $\alpha_6\beta_4$ is found in hemidesmosomes where it plays an essential role in their organization (10, 11). This integrin can also interact with F-actin and promote the migration of invasive carcinoma cells (12, 13).

The intracellular portion of the β_4 subunit is much larger (1,000 amino acids) than that of all the other known β subunits, and it does not exhibit apparent sequence homology with them (14–16). Increasing evidence indicates that the ability of $\alpha_6\beta_4$ to regulate cell proliferation, motility, and invasion is dependent upon signal transduction events that are mediated by the β_4 cytoplasmic domain (17–19). Of particular relevance to cancer, $\alpha_6\beta_4$ has been implicated in carcinoma invasion (19–21) through its ability to activate PI3K (19). Moreover, the ability of $\alpha_6\beta_4$ to promote invasion is dependent upon the β_4 cytoplasmic domain. The importance of $\alpha_6\beta_4$ in malignancy is also indicated by the finding that its expression correlates with the progression of squamous, ovarian, thyroid, gastric, and colorectal carcinomas (22–27).

Function and signaling properties of $\alpha_6\beta_4$ in carcinoma cells are influenced by its association with growth factor receptors. Specifically, we demonstrated that $\alpha_6\beta_4$ co-immunoprecipitates with ErbB-2 in human mammary and ovarian carcinoma cell lines and that ligation of this integrin increases ErbB-2 phosphorylation (28). Moreover, we also observed that overexpression of ErbB-2 and $\alpha_6\beta_4$ in NIH3T3 cells increased their invasive capacity (28). In the present study, we sought to identify the portion of β_4 involved in the interaction with ErbB-2 and possibly involved in the development of a more aggressive phenotype. With this aim, we generated different NIH3T3 transfectants that concomitantly overexpress ErbB-2 oncogene and wild type or deletion mutants of β_4 integrin. *In vitro* invasion assays demonstrated that the portion of β_4 protein involved in the invasive capacity resides in 329 cytoplasmic residues between the amino acids 824 and 1183. Biochemical analysis indicated that ErbB-2/β_4 interaction is abolished uniquely when the entire cytoplasmic domain of β_4 is deleted. Thus, we were able to exclude that the interaction of ErbB-2 and β_4 is responsible for increased malignancy of NIH3T3/ErbB-2/β_4 cells. The study of the mechanisms by which β_4 cooperates with ErbB-2 to promote increased malignancy showed that the $\alpha_6\beta_4$ integrin activates the PI3K pathway when both ErbB-2 and β_4 are overexpressed.

EXPERIMENTAL PROCEDURES

Cell Lines and cDNA Constructs—NIH3T3 parental cell line and NIH3T3 cells stably transfected with the human ErbB-2 cDNA (29) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and glutamine (Life Technologies, Inc.). NIH3T3 cells and ErbB-2-transduced NIH3T3 cells were transfected, by electroporation, with the pRC/CMV expression

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This paper is available on line at http://www.jbc.org

vector carrying the wild type human β_4 integrin subunit and truncated β_4 cDNAs (10, 17). Filippo Giancotti (Memorial Sloan-Kettering Cancer Center, New York) kindly provided the cDNAs encoding the full-length and truncated β_4 molecules. Selection of neomycin-positive clones was carried out using 500 μg/ml of G418 (Life Technologies, Inc.).

Antibodies—The rat monoclonal antibody (mAb)[1] 439-9B and the mouse mAb 450-11A to the human β_4 and the rat mAb 135-13C to α_6 integrin subunits, respectively, were purified as described previously (21, 30). Tom Carey (University of Michigan, Ann Arbor, MI) kindly donated the anti-human β_4 integrin mAb A9 (31). The anti-mouse β_4 integrin mAb 346-11A was prepared and purified from ascitic fluid and used as negative control (32). Purified anti-mouse IgG was from Cappel (Durham, NC). The mouse mAbs to the human ErbB-2 protein used in Western blotting experiments were from Transduction Laboratories (Lexington, KT) or from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated anti-mouse or anti-rabbit IgG were from Bio-Rad. The anti-phosphotyrosine (anti-Tyr(P)) mouse mAb 4G10 was from Upstate Biotechnology, Inc. (Upstate Biotechnology Inc., Lake Placid, NY).

Affinity Chromatography—Anti-human and anti-mouse β_4 integrin mAbs 439-9B and 346-11A were purified by high performance liquid chromatography and cross-linked to activated immune affinity supports Affi-Gel 10/15 (Bio-Rad). In brief, 4 mg of purified mAb was combined with 1 ml of Affi-Gel in 0.1 M buffer carbonate, pH 8.5, at 4 °C for 4 h. The beads were then washed with Tris-HCl 0.1 M, pH 8, and suspended in PBS containing 0.03% sodium azide (NaN_3). NIH3T3 transfectants were lysed in 20 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, aprotinin (5 μg/ml), leupeptin (10 μg/ml), and pepstatin A (4 μg/ml). Repeated experiments were also performed in the presence of 10 mM CHAPS (Pierce), instead of 1% Nonidet P-40, to disrupt nonspecific protein-protein interactions. Lysates were clarified by centrifugation, and the immune complexes were purified by affinity chromatography using mAbs cross-linked to Affi-Gel (bead-conjugated antibodies).

Immunocomplexes and Total Cell Lysates—Immunoprecipitations from cells labeled with lactoperoxidase and carrier-free ^{125}I showed that the antibodies recognize the appropriate molecules (data not shown). In brief, as described previously (21), 2×10^6 cells were labeled with 1 mCi of ^{125}I in the presence of 10 μl of lactoperoxidase (2 mg/ml in 50% of glycerol) (Calbiochem, La Jolla, CA) and 5 μl of a 1:1000 dilution of H_2O_2 (30%). After labeling, cells were washed with PBS and solubilized in lysis buffer containing 5 mg/ml BSA, 1% Nonidet P-40, 1 mM NaN_3, 1 mM phenylmethylsulfonyl fluoride (Sigma), 5 μg/ml leupeptin, 10 μg/ml aprotinin (Sigma), and 10 mM EDTA. The lysates were clarified by centrifugation (30,000 $\times$ *g*) for 3 h at 4 °C and solubilized proteins (1×10^7 cpm) were immunoprecipitated. The immunoprecipitates were analyzed by SDS-PAGE, and autoradiography was performed with X-Omat RP film (Kodak). Direct immunoprecipitations were performed using primary antibodies collected with 50 μl of Protein G-agarose beads (Pierce) suspended in lysis buffer (50% v/v). Total cell lysates were added to the bead-conjugated antibodies, and protein complexes were washed at 4 °C in lysis buffer, boiled, and analyzed by SDS-PAGE.

Kinase Assay—To assay PI3K activity, after serum starvation for 24 h the cells were washed and lysed in 10 mM Hepes, pH 7.5, 0.15 M NaCl, 10% glycerol in the presence of protease and phosphatase inhibitors. Nuclei were removed by centrifugation at 12,000 $\times$ *g* for 15 min at 4 °C. After lysis, aliquots of cell extracts containing equivalent amounts of protein were incubated overnight at 4 °C with anti-Tyr(P) mAb 4G10 (Upstate Biotechnology, Inc.) and protein G (Pierce). The beads were washed twice with lysis buffer (10 mM Hepes, pH 7.5, 0.15 M NaCl, 10% glycerol, and 1% Nonidet P-40); twice with 0.5 M LiCl; twice with 10 mM Hepes, pH 7.5, 0.15 M NaCl, and 0.2% Nonidet P-40; and once with 10 mM Hepes, pH 7.5, 0.15 M NaCl. After removal of the last wash, the beads were suspended in 30 μl of 30 mM Hepes, pH 7.5, and 30 μl of kinase buffer containing 10 μg (20 μl) of L-α-phosphatidylinositol from bovin liver (Sigma) sonicated in 10 mM Hepes and 1 mM EDTA, 80 μM ATP (Roche Molecular Biochemicals), 20 μCi of [γ-^{32}P]ATP (6000 Ci/mmol), 10 mM $MgCl_2$, and 400 μM adenosine (Sigma) and incubated for 25 min at room temperature. The reaction was stopped by the addition of 100 μl of 1 M HCl, and 200 μl of 1:1 mixture of chloroform and methanol were added. The organic phase was then washed twice with 300 μl of 1:1 mixture of methanol and HCl. The lipid extracts (20 μl of each reaction) were then resolved by thin layer chromatography plates (TLC Silica gel) (Merck) in chloroform, methanol, and ammonium hydroxide. Dried TLC sheets were developed by autoradiography.

Western Blot Analysis—Total cell lysates or immune complexes obtained by affinity chromatography or indirect immunoprecipitation were analyzed by SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad) or Immobilon-P transfer membrane (Millipore, Bedford, MA). The blots were probed with the following antibodies: 2 μg/ml of anti-ErbB-2 mAb (Clone 3B5) or 2 μg/ml of purified mouse anti-human c-Myc mAb (Clone 9E10). Filters were washed and developed with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). Autoradiographies were performed with Hyperfilm ECL (Amersham Pharmacia Biotech).

Flow Cytometry—The expression levels of ErbB-2 and $\alpha_6\beta_4$ integrin receptors were detected by flow cytometric analysis of stained cells. Cells harvested using citrate saline buffer (0.134 M KCl, 0.015 M sodium citrate) were washed twice with cold PBS containing 0.002% EDTA and 10 mM NaN_3 (washing buffer). Samples of 1×10^6 cells were incubated for 1 h at 4 °C with saturating concentrations of primary antibodies diluted in PBS containing 0.5% BSA. Control cells were incubated with unrelated antibodies. Cells were then washed three times with washing buffer (PBS containing 0.5% BSA) and incubated for 1 h at 4 °C with 50 μl of fluorescein isothiocyanate-conjugated secondary antibodies ($F(ab')_2$ (Cappel, West Chester, PA)) diluted 1:20 in PBS/BSA. After three washes, the cells were suspended in 1 ml of washing buffer. Cell suspensions were analyzed by a flow cytometer (Epics XL analyzer, Coulter Corporation, Miami, FL) after addition of 5 μl of propidium iodide (1 mg/ml stock solution) to exclude nonviable cells. At least 1×10^4 cells/sample were analyzed.

Chemoinvasion Assays—Chemoinvasion assays were carried out in a Boyden chamber as described (33). In brief, 8-mm polycarbonate filters (Nucleopore, Concorezzo, Italy) were coated with Matrigel kindly provided by Dr. A. Albini (Genova, Italy). Optimal Matrigel concentration (12.5 μg/filter from a 250 μg/ml dilution of Matrigel in distilled, cold water) was accurately determined in preliminary experiments. The cells harvested by trypsin-EDTA treatment were washed with serum free Dulbecco's modified Eagle's medium supplemented with 0.1% BSA, and 5×10^5 cells were layered on the top well of a Boyden chamber. The chambers were incubated at 37 °C, 5% CO_2 for 8 h in the presence of Balb/3T3 conditioned medium (added as chemo-attractant) or in the presence of 0.1% BSA (added as negative control). In some assays, the cells were preincubated for 30 min before addition to the Matrigel-coated wells with wortmannin (Biomol, Plymouth Metting, PA). Cells migrated on the lower surface of the filters were fixed in ethanol and stained with Toluidine blue. Five independent fields were counted at 160× with a Zeiss microscope. Each assay was carried out in quadruplicate and repeated at least three times. The ability of the cells to adhere to the filters was verified by staining the upper side of the filters.

RESULTS

The Cytoplasmic Domain of β4 Protein Influences the Metastatic Propensity of NIH3T3/ErbB-2-transformed Cells—We previously demonstrated that the expression of the β_4 integrin subunit in NIH3T3 cells, transformed by the ErbB-2 oncogene, stimulates their *in vitro* invasion (28). To identify specific domains in the β_4 subunit that confer this invasive potential, we expressed deletion mutants of β_4 in NIH3T3/ErbB-2 cells (Fig. 1). These deletion mutants lacked either all (L) or portions (B, C, and D) of the β_4 cytoplasmic domain. In addition, we used an ecto-domain mutant (F) in which most of the extracellular domain of β_4 was replaced with a c-Myc tag. In agreement with data previously reported (10), all of these mutants were highly expressed on the cell surface of the transfected cells (data not shown). Clones of these transfectants expressing comparable surface expression of the β_4 subunit were chosen for functional and biochemical analyses. The expression levels of β_4 protein corresponding to F deletion mutant were assessed by Western blot analysis using a mouse anti-human c-Myc antibody (Fig. 2). Immunoprecipitation of surface-labeled proteins confirmed the expression of wild type and truncated β_4 proteins on the cell surface of selected clones (data not shown).

To identify specific domains within the β_4 subunit that are

[1] The abbreviations used are: mAb, monoclonal antibody; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; PI3K, phosphatidylinositol 3-kinase.

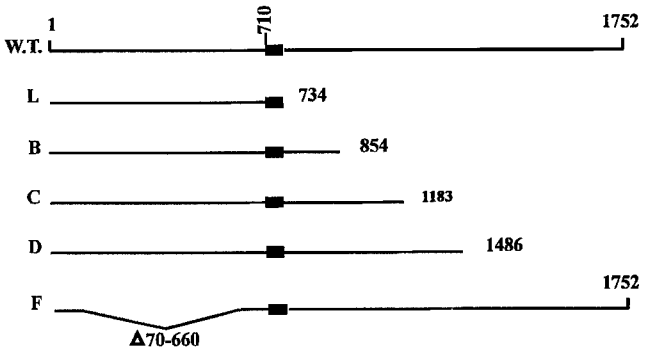

FIG. 1. **Schematic representation of wild type and mutated β_4 cDNAs.** ■, transmembrane domain; amino acids 1–710, extracellular domain; amino acids 734–1752, cytoplasmic domain; *Δ70–660*, extracellular domain deletion. Deletions in the cytoplasmic domain are indicated by the amino acids where the molecules were truncated.

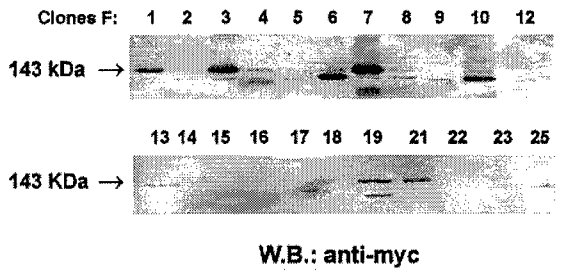

FIG. 2. **Western blot analysis using a mouse anti-human c-Myc antibody of the clones obtained after selection of NIH3T3/ErbB-2 cells transfected with deleted β_4 F cDNA.** The molecular size of β_4 F transfectants (kilodaltons) is indicated by an *arrow*. *W.B.*, Western blot.

necessary for the ability of $\alpha_6\beta_4$ to promote invasion, the NIH3T3/ErbB-2 cells that expressed the β_4 deletion mutants were assayed for their ability to invade Matrigel in a standard chemoinvasion assay (Table I). At least two clones from each of the NIH3T3/ErbB-2/β_4 transfectant were used for this purpose. In agreement with our previous finding (28), expression of the wild type β_4 subunit increased invasion of NIH3T3/ErbB-2 cells by 2.5-fold. Interestingly, expression of the β_4 C, D, and F mutants resulted in a similar increase in invasion (Table I). However, expression of the β_4 L and B mutants did not enhance the invasiveness of NIH3T3/ErbB-2 cells. Two important conclusions can be drawn from these data. First, the extracellular domain of β_4 does not appear to be necessary for $\alpha_6\beta_4$ to stimulate invasion based on the results obtained with the β_4 F mutant. Second, a portion of the β_4 cytoplasmic domain between amino acids 854 (the site of the β_4 B deletion) and 1183 (the site of the β_4 C deletion) is critical for the ability of the $\alpha_6\beta_4$ integrin to stimulate invasion. However, the β_4 mutated proteins we used contain very large deletions, leaving open the possibility for the domains of the β_4 protein, upstream of the 854 amino acid, to stimulate invasion but which at the same time require cooperation with the other downstream domains. We also cannot exclude that such deletions, causing conformational changes of the molecules, could prevent their interaction with intermediate signaling molecules responsible for stimulating invasion.

Interaction of β_4 with ErbB-2 Is Abrogated by the Lack of β_4 Cytoplasmic Domain—Given our previous demonstration of a physical association between $\alpha_6\beta_4$ and ErbB-2, we attempt to determine whether this association is necessary for the ability of $\alpha_6\beta_4$ and ErbB-2 to promote invasion. Specifically, we examined the ability of ErbB-2 to co-immunoprecipitate with $\alpha_6\beta_4$ heterodimers that contained specific deletions in the β_4 subunit. To this purpose, these $\alpha_6\beta_4$ heterodimers were purified by

TABLE I
Chemoinvasive ability of the NIH3T3 transfectants

NIH3T3 transfectants were assayed for their ability to invade Matrigel in the absence or presence of the PI3K inhibitor wortmannin (100 nM. Fibroblast-conditioned serum-free medium was added in the lower compartment of the Boyden Chamber and used as chemoattractant. The data shown are from two individual clones of each transfectant and are the mean values ± S.D. of three experiments done in quadruplicate. Statistical significance (P^*) was evaluated according to T-test comparing the median values of NIH3T3/ErbB-2 cells/field *versus* themselves or NIH3T3/ErbB-2/β4 transfectants cells/field in absence or presence of wortmannin. ND, not determined.

Cell lines	Wortmannin			
	–		+	
	Cells/field	P^*	Cells/field	P^*
NIH3T3/ErbB-2	48 ± 17		50 ± 15	0.886
NIH3T3/ErbB-2/β_4 wild type	112 ± 12	0.006	<1	
NIH3T3/ErbB-2/β_4 L	42 ± 8	0.61	ND	
NIH3T3/ErbB-2/β_4 B	56 ± 5	0.478	ND	
NIH3T3/ErbB-2/β_4 C	108 ± 7	0.005	<1	
NIH3T3/ErbB-2/β_4 D	112 ± 12	0.011	<1	
NIH3T3/ErbB-2/β_4 F	130 ± 15	0.003	<1	

affinity chromatography using either 439-9B mAb, which recognizes an epitope present in the extracellular domain of β_4 or 450-11A mAb, which recognizes an epitope in the β_4 cytoplasmic domain. As negative control, we used 346-11A mAb, which is specific for mouse β_4. The presence of ErbB-2 in the β4 integrin immune complexes was determined by immunoblotting. Using this approach, we detected the 185-kDa ErbB-2 protein in the β_4 immune complexes obtained from extracts of NIH3T3/ErbB-2 cells that expressed the intact $\alpha_6\beta_4$ heterodimer (Fig. 3, *lane 1*), as expected (28). In addition, an ErbB-2/β_4 complex was detected in extracts obtained from clones 19 and 21 of cells expressing the extracellular domain deletion of β_4 (β_4 F) (Fig. 3). In these immune complexes, purified by an anti-β_4 mAb specific for the cytoplasmic domain of the molecule, the anti-ErbB-2 mAb was able to detect a 185-kDa protein (Fig. 3, *lanes 2* and *3*). Moreover, the co-immunoprecipitation of ErbB-2 and β_4 F truncated protein was detected after reprobing the same blot with an anti-c-Myc mAb (Fig. 3, *lanes 5* and *6*). The same anti-c-Myc antibody did not recognize the β_4 wild type protein (*lane 4*). Similar experiments performed using extracts from NIH3T3/ErbB-2 cells expressing truncated proteins β_4 C (clones 9 and 17) and D (clones 3 and 25) revealed that all these β_4 truncated proteins co-immunoprecipitate with the ErbB-2 protein (Fig. 4, *lanes 1, 3, 5,* and *7*, respectively). The mAb specific for the mouse β_4 integrin (negative control) did not co-immunoprecipitate ErbB-2 from the same extracts (Fig. 4, *lanes 2, 4, 6,* and *8*). Fig. 5 shows immune complexes from extracts of NIH3T3/ErbB-2 cells transfected with truncated proteins β_4 B (clones 11 and 14) and L (clones 5 and 4). The truncated β_4 B protein still co-precipitated with ErbB-2 (Fig. 5, *lanes 3* and *4*), whereas β_4 L protein, which was deleted from the entire cytoplasmic domain, did not co-precipitate with ErbB-2 (Fig. 5, *lanes 6* and *7*). As controls, the ErbB-2 protein was detected in immune complexes derived from lysates of NIH3T3/ErbB-2 cells expressing wild type β_4 protein (Fig. 5, *lane 2*) but not from lysates of NIH3T3/ErbB-2 cells (Fig. 5, *lane 1* and *5*). These data show that only the deletion of the entire β_4 cytoplasmic domain abrogates co-immunoprecipitation of ErbB-2 and β_4. Moreover, the finding that the β_4 F protein, which is unable to form $\alpha_6\beta_4$ heterodimers (10) but is still able to retain the ability to co-precipitate with ErbB-2 indicates that the α_6 subunit is not involved in the ErbB-2/β_4 interaction.

Cooperativity between $\alpha_6\beta_4$ and ErbB-2 Is Required to Activate PI3K—The activation of PI3K by the $\alpha_6\beta_4$ integrin has

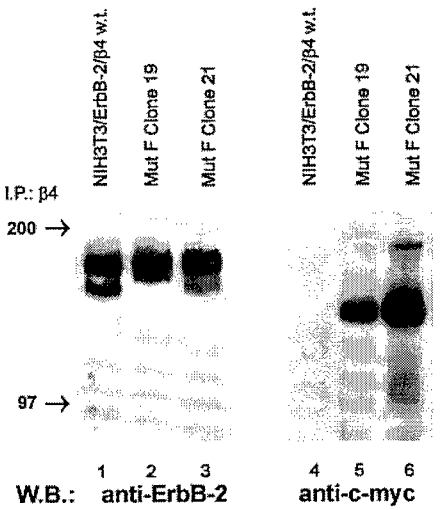

FIG. 3. **Association of β_4 mutant F with ErbB-2.** Total cell lysates from NIH3T3/ErbB-2/β_4 wild type (*lanes 1* and *4*) and NIH3T3/ErbB-2/β_4 mutant F cells (clones 19 and 21) (*lanes 2, 3, 5,* and *6*) were incubated with bead-conjugated anti-human β_4 mAb 439-9B. Immune complexes were analyzed by SDS-PAGE and probed as shown in the *left panel* with a mouse anti-human ErbB-2 mAb followed by chemiluminescence detection with an anti-mouse IgG peroxidase-conjugated antibody. The *right panel* shows the immunoblot on the same filter with a mouse anti-human c-Myc antibody. Molecular sizes (kilodaltons) are indicated. *W.B.*, Western blot.

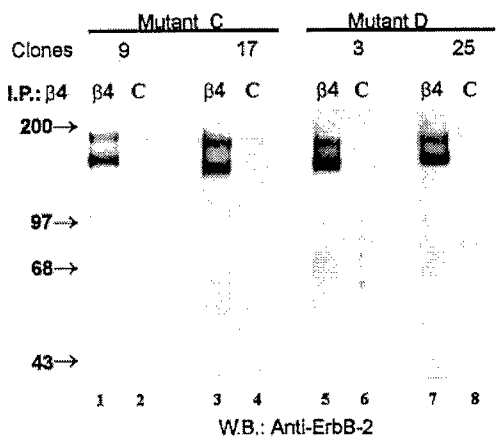

FIG. 4. **Association of the β_4 mutant C and D molecules with ErbB-2.** Total cell lysates from NIH3T3/ErbB-2/β4 mutant C and D cells were incubated with bead-conjugated anti-human β4 antibody 439-9B (*lanes 1, 3, 5,* and *7*) or, as negative control, anti-mouse β4 antibody 346-11A (*lanes 2, 4, 6,* and *8*). Immune complexes were analyzed by SDS-PAGE and probed with a mouse anti-human ErbB-2 mAb followed by chemiluminescence detection with anti-mouse IgG peroxidase-conjugated antibody. Molecular sizes (kilodaltons) are indicated. *W.B.*, Western blot.

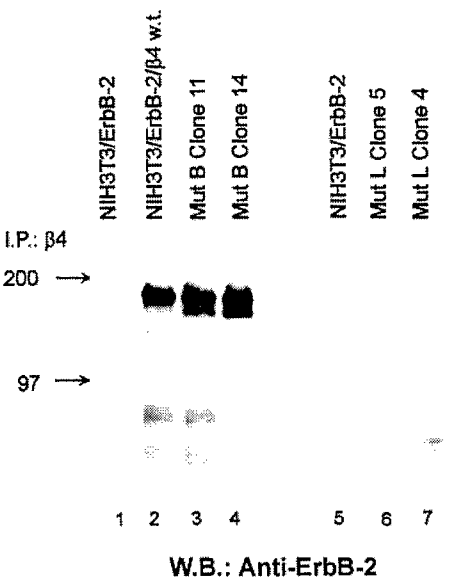

FIG. 5. **Association of the β_4 mutant B and L molecules with ErbB-2.** Total cell lysates from NIH3T3/ErbB-2 (*lanes 1* and *5*), from NIH3T3/ErbB-2/β_4 wild type (*lane 2*), from NIH3T3/ErbB-2/β_4 mutant B (clone 11 and 14) (*lanes 3* and *4*), and from NIH3T3/ErbB-2/β_4 mutant L (clones 5 and 4) (*lanes 6* and *7*) cells were incubated with bead-conjugated anti-human β_4 439-9B antibody. Immune complexes were analyzed by SDS-PAGE and probed with a mouse anti-human ErbB-2 mAb followed by chemiluminescence detection with an anti-mouse IgG peroxidase-conjugated antibody. Molecular sizes (kilodaltons) are indicated. *W. B.*, Western blot.

been shown to promote the invasion of carcinoma cells (19). Based on our finding that the overexpression of $\alpha_6\beta_4$ increases the invasiveness of NIH3T3 cells transformed by ErbB-2, we examined the influence of these two surface receptors on PI3K activation. As a prelude to these experiments, we established the involvement of PI3K in the invasion of the NIH3T3/ErbB-2/β_4 transfectants using wortmannin. In agreement with previous findings (19, 34), we found that wortmannin inhibited the invasion of NIH3T3 cells expressing either the intact $\alpha_6\beta_4$ heterodimer or the various β_4 deletion mutants that are still able to increase invasiveness (Table I). Interestingly, we also found that wortmannin did not modify the invasive capacity of NIH3T3/ErbB-2 cells (Table I), confirming the observation that PI3K pathway is involved in β_4-dependent invasive capacity. The fact that wortmannin completely abolishes the invasiveness of NIH3T3/ErbB-2/β_4 cells rather than reducing it to the level of parental cell line might be explained by the assumption that the activation of PI3K in NIH3T3/ErbB-2 cells after β_4 expression supersedes other signaling pathways responsible for the invasion of these cells. Based on this hypothesis, it is reasonable to think that wortmannin-mediated inhibition of PI3K activation should abolish the invasive capacity.

To assay PI3K activity, extracts obtained from NIH3T3/ErbB-2 transfectants were immunoprecipitated with an anti-Tyr(P) mAb to capture the activated population of PI3K, and the immunoprecipitates were assayed for their ability to phosphorylate L-α-phosphatidylinositol. Constitutive PI3K activity was undetectable in NIH3T3 parental cells and also in NIH3T3 cells overexpressing either the β_4 or the ErbB-2 proteins alone (Fig. 6). However, PI3K activation was evident when β_4 protein was co-expressed with ErbB-2 (Fig. 6). These results indicate that both $\alpha_6\beta_4$ and ErbB-2 expression are required for constitutive PI3K activation in NIH3T3 cells. Furthermore, these data indicate that in the NIH3T3/ErbB-2 cellular context a correlation exists between the β_4-dependent invasive capacity and the β_4-induced PI3K activity. Moreover, the availability of the β_4 deletion mutants enabled us to identify specific domains within the β_4 subunit that are required for PI3K activation and to compare these domains with those that are implicated in invasion based on our results. Cell extracts of the NIH3T3/ErbB-2 transfectants that expressed the β_4 C, D, and F truncated proteins exhibited levels of PI3K activation comparable with that of wild type β_4 (Fig. 7). In contrast, NIH3T3/ErbB-2 transfectants expressing the β_4 L and B proteins that did not enhance the invasiveness of NIH3T3/ErbB-2 cells (Table I) showed minimal activation of PI3K (Fig. 7). To establish a possible link between the formation of the receptor-integrin complex and PI3K activity, we checked the presence of PI3K in the complex. However, using different experimental conditions, we were never able to detect PI3K in ErbB-2/β_4 complex (data not shown).

We then questioned whether antibody-mediated ligation of

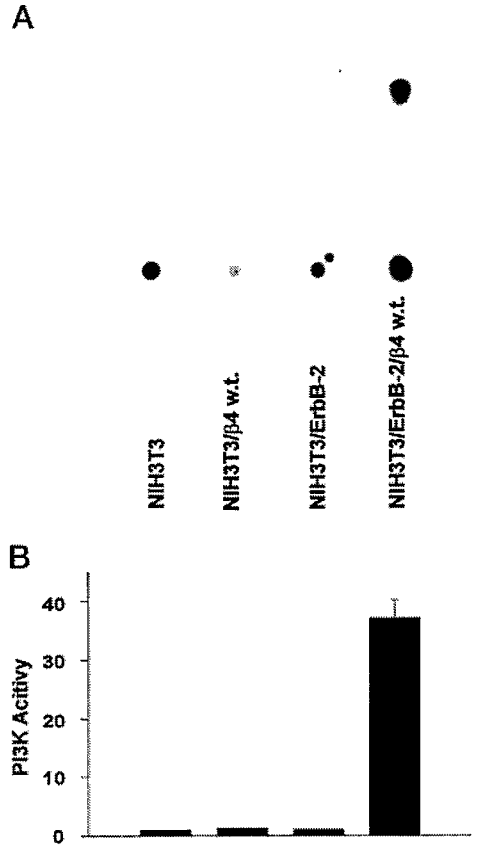

FIG. 6. **Analysis of PI3K activity on NIH3T3, NIH3T3/β_4, NIH3T3/ErbB-2, and NIH3T3/ErbB-2/β_4 clones.** *A*, aliquots of cell extracts derived from the parental cell line NIH3T3 (*lane 1*) and NIH3T3 clones (*lanes 2–4*) containing equivalent amounts of protein were incubated with the anti-phosphotyrosine antibody 4G10 and protein G-Sepharose overnight at 4 °C. After washing, the beads were resuspended in kinase buffer for 20 min at room temperature in presence of L-α-phosphatidylinositol. The phosphorylated lipids were resolved by thin layer chromatography. *B*, the amount of radiolabeled lipids was determined by densitometry. The data shown are the mean values ± S.D. from three separate experiments.

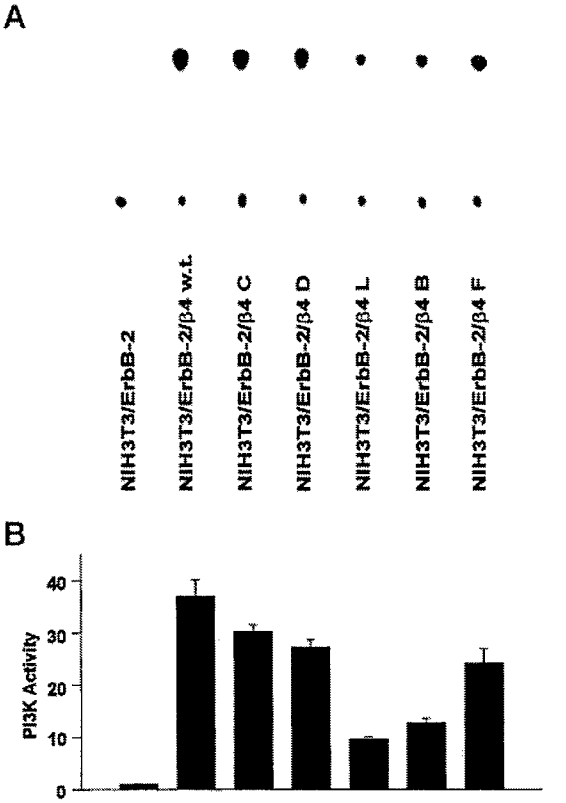

FIG. 7. **Analysis of PI3K activity on NIH3T3/ErbB-2, NIH3T3/ErbB-2/β_4 wild type, and/or NIH3T3/ErbB-2 deleted β_4 molecules C, D, L, B, and F.** *A*, aliquots of cell extracts containing equivalent amounts of protein were incubated with the anti-Tyr(P) mAb and protein G-Sepharose overnight at 4 °C. After washing, the beads were resuspended in kinase buffer for 20 min at room temperature in the presence of L-α-phosphatidylinositol. The phosphorylated lipids were resolved by thin layer chromatography. *B*, the amount of radiolabeled lipids was determined by densitometry. The data shown are the mean values ± S.D. from three separate experiments.

$\alpha_6\beta_4$ could augment PI3K activation. To this purpose, we used a mAb specific for the α_6 integrin subunit because it allowed us to compare activation of PI3K by the $\alpha_6\beta_1$ and $\alpha_6\beta_4$ integrins. However, NIH3T3 cells express $\alpha_6\beta_1$ but not $\alpha_6\beta_4$. We observed that antibody-mediated ligation of the α_6 subunit ($\alpha_6\beta_1$) in the NIH3T3/ErbB-2 cells did not stimulate PI3K activation (Fig. 8). However, ligation of the α_6 subunit in the NIH3T3/ErbB-2/β_4 transfectants (primarily $\alpha_6\beta_4$) stimulated PI3K activity (Fig. 8). Finally, we asked whether wortmannin could affect PI3K activity on NIH3T3/ErbB-2/β_4 cells. In agreement with previous findings (35), we found that treatment with wortmannin is able to reduce PI3K activity by 90% in these cells (Fig. 9), supporting the effect of the complete inhibition of invasiveness that was obtained after treatment of NIH3T3/ErbB-2/β_4 with this inhibitor (Table I). These data further indicate that PI3K activation in NIH3T3/ErbB-2 cells is strictly dependent upon β_4 expression and that in these cells ligand activation of the $\alpha_6\beta_1$ receptors cannot substitute β_4-dependent signals to generate PI3K activity.

DISCUSSION

Recent studies have implicated a key role of the $\alpha_6\beta_4$ integrin in carcinoma invasion and progression by a mechanism that involves its ability to activate PI3K (19). In addition, we have recently provided evidence that $\alpha_6\beta_4$ is able to associate with ErbB-2 (28), a growth factor receptor associated with aggressive breast carcinomas (36). In the current study, we defined the mechanism by which $\alpha_6\beta_4$ and ErbB-2 cooperate to promote invasion using NIH3T3 cells as a model system. We found that the expression of both $\alpha_6\beta_4$ and ErbB-2 in these cells is required to enhance their PI3K-dependent invasion through Matrigel. More importantly, expression of both $\alpha_6\beta_4$ and ErbB-2 is also required for the activation of PI3K, an observation that reinforces the importance of PI3K in invasion and has significant implications on how this lipid kinase is activated. The expression of β_4 deletion mutants in NIH3T3/ErbB-2 cells enabled us to identify a specific region within the β_4 cytoplasmic domain (329 amino acids) that is essential for the ability of $\alpha_6\beta_4$ to stimulate invasion. An important finding, in this context, is that neither the extracellular domain of the β_4 subunit nor $\alpha_6\beta_4$ heterodimerization are needed for $\alpha_6\beta_4$ enhancement of invasion. Altogether, our results indicate that the β_4 cytoplasmic domain cooperates with ErbB-2 to activate PI3K and stimulate invasion.

Our finding that the extracellular domain of the β_4 subunit is not required for the stimulation of invasion and activation of PI3K in NIH3T3 cells is interesting in view of recent reports showing the ability of $\alpha_6\beta_4$ to promote invasion and chemotaxis, which can occur independently of $\alpha_6\beta_4$ ligation (19, 13). From these observations one can infer that the large β_4 cytoplasmic domain can either initiate signaling events autonomously or influence the function of other receptors such as ErbB-2. This latter possibility is supported by our observation that the c-Myc/β_4 cytoplasmic domain chimera is able to associate with ErbB-2 in the absence of the β_4 extracellular domain.

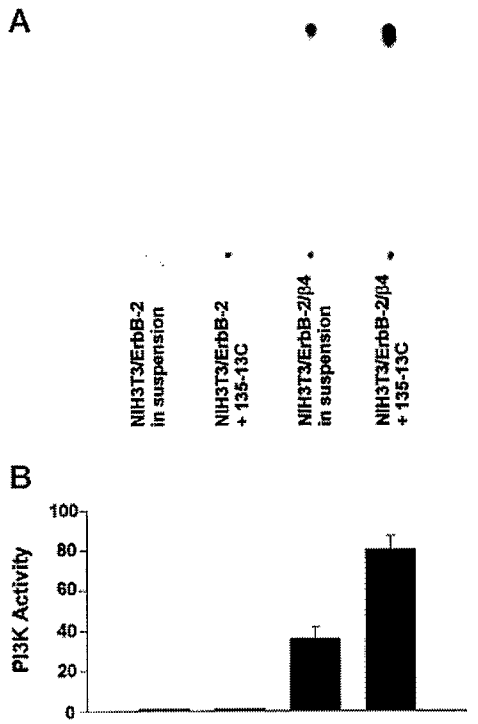

FIG. 8. **Analysis of PI3K activity on NIH3T3/ErbB-2 and NIH3T3/ErbB-2/β_4 wild type cells.** *A*, NIH3T3/ErbB-2 and NIH3T3/ErbB-2/β_4 cells were maintained in suspension (*lanes 1* and *3*) or plated on dishes coated with the anti-α6 specific antibody 135-13C (*lanes 2* and *4*) and allowed to adhere. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with the anti-Tyr(P) mAb and protein G-Sepharose overnight at 4 °C. After washing, the beads were resuspended in kinase buffer for 20 min at room temperature in the presence of L-α-phosphatidylinositol. The phosphorylated lipids were resolved by thin layer chromatography. *B*, densitrometric analysis show that the integrin clustering induces a 2-fold increase of PI3K activation in NIH3T3/ErbB-2/β_4 cells (36.2 ± 6 *versus* 80.4 ± 7). The standard deviation of two separate experiments is indicated.

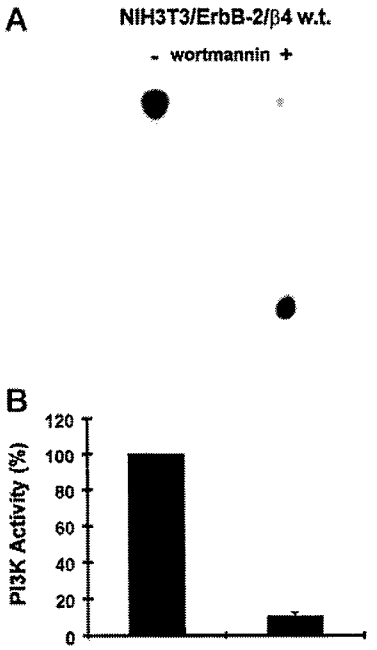

FIG. 9. **Analysis of PI3K activity on NIH3T3/ErbB-2/β_4 cells.** *A*, NIH3T3/ErbB-2/β_4 cells were maintained in adhesion (*lane 1*) or treated with wortmannin. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with the anti-Tyr(P) mAb and protein G-Sepharose overnight at 4 °C. After washing, the beads were resuspended in kinase buffer for 20 min at room temperature in presence of L-α-phosphatidylinositol. The phosphorylated lipids were resolved by thin layer chromatography. *B*, the percentage of radiolabeled lipids inhibition was determined by densitometry.

Although, the mechanisms by which the β_4 cytoplasmic domain is able to enhance invasion and interact with ErbB-2 are not presently known, the recent findings that the β_4 cytoplasmic is capable of self-association (37) suggest that clustering of this domain could initiate signaling events in the absence of extracellular domain ligation. The independence of the β_4 cytoplasmic domain is also supported by the finding that this domain is sufficient for direct localization of β_4 into adhesive sites (6).

Although the PI3K activation by $\alpha_6\beta_4$ has been shown to be relevant for invasion, the mechanism by which this or other integrins activate this lipid kinase is not known. In fact, the β_4 cytoplasmic domain lacks the YMXM consensus motif for binding the regulatory p85 subunit of PI3K via SH2 domains. This motif is present in several growth factor receptors that activate PI3K (35, 38). It is clear from our data that both $\alpha_6\beta_4$ and ErbB-2 are required to induce activation of PI3K. Our finding that the expression of ErbB-2 alone is not sufficient to activate PI3K is in agreement with the report that ErbB-2, by itself, does not recruit PI3K but activates it only after heregulin stimulation and ErbB-2/ErbB-3 dimerization (39–41). Given the fact that heregulin was not present in our experiments, the conclusion can be drawn that the association of $\alpha_6\beta_4$ with ErbB-2 mimics the ErbB-2/ErbB-3 dimerization that is required for PI3K activation. The fact that ErbB-2 also lacks the consensus motif for p85 binding (42) suggests that the mechanism by which $\alpha_6\beta_4$ and ErbB-2 cooperate to activate PI3K involves their synergistic activation of signaling intermediates. The identification of the involvement of such signaling intermediates should increase our understanding of PI3K activation and invasion markedly.

Interestingly, expression of both $\alpha_6\beta_4$ and ErbB-2 in NIH3T3 resulted in the constitutive activation of PI3K in the absence of either $\alpha_6\beta_4$ ligation or heregulin stimulation. This result reinforces our hypothesis that $\alpha_6\beta_4$, the β_4 cytoplasmic domain in particular, is able to initiate signaling events in the absence of receptor ligation and clustering. This hypothesis is supported by the recent finding that activation of a cAMP-dependent phosphodiesterase by $\alpha_6\beta_4$ is independent of $\alpha_6\beta_4$ ligation (13). Moreover, an increase in the constitutive activation of PI3K upon expression of $\alpha_6\beta_4$ in a breast carcinoma cell line has been observed (19). In agreement with our findings, constitutive activation of PI3K could be enhanced by antibody-mediated clustering of $\alpha_6\beta_4$ or by attachment to laminin. It is also apparent from our findings that a threshold of PI3K activation exists for the stimulation of invasion. Specifically, we observed significantly less PI3K activity upon expression of the β_4 L and B mutants in comparison to the wild type and β_4 C, D, and F mutants (Fig. 7), and this activity correlated with the ability of these mutants to stimulate invasion.

An important issue that had not been addressed prior to our study is the identification of a specific region of the large β_4 cytoplasmic domain that is essential for its ability to stimulate invasion and activate PI3K. Indeed, the β_4 cytoplasmic domain has been implicated in many cellular functions including not only invasion and PI3K activation but also hemidesmosome assembly (19, 43), dynamic interactions with F-actin (12), as well as cell proliferation (17) and apoptosis (44, 45). In addition, evidence has been presented for the binding of specific molecules to the β_4 cytoplasmic domain including the adaptor protein Shc and the cytoskeletal-associated protein plectin or HD-1 (46, 47). To date, reasonably good evidence exists for specific sites within the β_4 cytoplasmic domain involved in hemidesmosome assembly and plectin binding (36). However, the β_4 domain involved in invasiveness, which we identified in

our study, does not strictly correspond to the β_4 domain able to interact with plectin in the stabilization of the hemidesmosomes.

In conclusion, we have identified a short portion of the β_4 protein sufficient to generate invasive capacity and found that ErbB-2 oncogene and β_4 protein cooperate to generate unexpected levels of PI3K activity, which is at least partially dependent upon signals generated from the β_4 cytoplasmic domain. These findings are relevant not only for the comprehension of malignant phenotype in transformed cells but also for the potential development of specific drugs.

Acknowledgments—We are grateful to Filippo Giancotti for providing us with β_4 deleted cDNAs, Adriana Albini for the Matrigel, and Tom Carey for the anti-β_4 A9 antibody. We are also particularly grateful to Robin Bachelder, Kathleen O'Connor, Isaac Rabinovitz, Leslie Shaw, and Silvia Soddu for helpful discussions.

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Brief Report

RhoA Function in Lamellae Formation and Migration Is Regulated by the α6β4 Integrin and cAMP Metabolism

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Abstract. Clone A colon carcinoma cells develop fan-shaped lamellae and exhibit random migration when plated on laminin, processes that depend on the ligation of the α6β4 integrin. Here, we report that expression of a dominant negative RhoA (N19RhoA) in clone A cells inhibited α6β4-dependent membrane ruffling, lamellae formation, and migration. In contrast, expression of a dominant negative Rac (N17Rac1) had no effect on these processes. Using the Rhotekin binding assay to assess RhoA activation, we observed that engagement of α6β4 by either antibody-mediated clustering or laminin attachment resulted in a two- to threefold increase in RhoA activation, compared with cells maintained in suspension or plated on collagen. Antibody-mediated clustering of β1 integrins, however, actually suppressed Rho A activation. The α6β4-mediated interaction of clone A cells with laminin promoted the translocation of RhoA from the cytosol to membrane ruffles at the edges of lamellae and promoted its colocalization with β1 integrins, as assessed by immunofluorescence microscopy. In addition, RhoA translocation was blocked by inhibiting phosphodiesterase activity and enhanced by inhibiting the activity of cAMP-dependent protein kinase. Together, these results establish a specific integrin-mediated pathway of RhoA activation that is regulated by cAMP and that functions in lamellae formation and migration.

Key words: carcinoma • protein kinase A • G-protein • phosphodiesterase • cytoskeleton

Introduction

The organization and remodeling of the actin cytoskeleton are controlled by the Rho family of small GTPases, which includes Rho, Rac, and cdc42. These proteins have been implicated in the formation of stress fibers, lamellipodia, and filopodia, respectively (reviewed in Hall, 1998). Although much of our knowledge on their function has been obtained from studies with fibroblasts, other activities for these Rho GTPases have been observed recently in cells of epithelial origin. For example, Rac and cdc42 are required to maintain apical–basal polarity in epithelia (Jou and Nelson, 1998). Rho, interestingly, has been implicated in membrane ruffling in epithelial cells (Nishiyama et al., 1994; Fukata et al., 1999), a process attributed to Rac in fibroblasts (Hall, 1998). These findings are of particular interest with respect to our understanding of epithelial cell migration. The migration and invasion of epithelial-derived carcinoma cells are important phenomena that require the involvement of Rho GTPases (Keely et al., 1997; Shaw et al., 1997; Yoshioka et al., 1998; Itoh et al., 1999). For these reasons, it is essential to define the factors that regulate the function of Rho GTPases in carcinoma cells and to characterize the mechanisms by which they contribute to the dynamics of migration. For example, although cell adhesion has been reported to activate RhoA (Barry et al., 1997; Ren et al., 1999), little is known about the involvement of specific integrins in adhesion-dependent RhoA activation or in the regulation of RhoA-dependent functions.

Recent studies by our group have highlighted a pivotal role for the integrin α6β4 in the migration and invasion of carcinoma cells, as well as in epithelial wound healing (Lotz et al., 1997; Rabinovitz and Mercurio, 1997; Shaw et al., 1997; O'Connor et al., 1998; Rabinovitz et al., 1999). Although it is well established that α6β4 functions in the formation and stabilization of hemidesmosomes (Borradori and Sonnenberg, 1996; Green and Jones, 1996), our findings revealed a novel role for this integrin in the formation of actin-rich cell protrusions at the leading edges of carcinoma cells and in the migration of these cells

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(Rabinovitz and Mercurio, 1997; Rabinovitz et al., 1999). Moreover, we demonstrated the importance of cAMP metabolism in these events (O'Connor et al., 1998). Given the recent interest in the participation of RhoA in migration, we examined the hypothesis that RhoA is essential for the formation of actin-rich cell protrusions, the migration of carcinoma cells and, more importantly, that the activity of RhoA is regulated by the α6β4 integrin. In addition, we assessed the involvement of cAMP metabolism in these events.

Materials and Methods

Cells and Antibodies

Clone A cells, originally isolated from a poorly differentiated colon adenocarcinoma (Dexter et al., 1979), were used in all experiments. For each experiment, adherent cells were harvested by trypsinization, rinsed three times with RPMI medium containing 250 μg/ml heat-inactivated BSA (RPMI/BSA), and resuspended in RPMI/BSA. Where indicated, cells were treated with 1 mM isobutylmethylxanthine (IBMX)[1] or 15 mM H-89 (Calibiochem-Novabiochem, Int.) for 15 min before use. The following antibodies were used in this study: MC13, mouse anti-β1 integrin mAb (obtained from Steve Akiyama, National Institutes of Health, Research Triangle Park, NC); K20, mouse anti-β1 integrin mAb (Immunotech); 439-9B, rat anti-β4 integrin mAb (obtained from Rita Falcioni, Regina Elena Cancer Institute, Rome, Italy); mouse anti-HA mAb (Roche Biochemicals); rabbit anti-RhoA polyclonal antibody (Santa Cruz Biotechnology); and anti-Rac1 (Transduction Laboratories).

To obtain expression of N19RhoA and N17Rac1, adherent cells were harvested using trypsin, rinsed with PBS, and suspended in electroporation buffer (20 mM Hepes, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4-$7H_2O$, 5 mM glucose). Cells were cotransfected with 1 mg of either pCS2-(n)β-gal or pGFP (green fluorescent protein) and 4 μg of either control vector or vector containing HA-tagged N19RhoA (provided from Alex Toker, Beth Israel Deaconess Medical Center, Boston, MA) or GST-tagged N17Rac1 (obtained from Margaret Chou, University of Pennsylvania) by electroporation at 250V and 500 μFd. Subsequently, cells were plated in complete growth medium containing 0.05% sodium butyrate and used for experiments 48 h after the initial transfection. Expression of the recombinant proteins was confirmed by concentrating extracts of transfected cells with an HA-specific mAb or glutathione-coupled beads and subsequent immunoblotting for RhoA or Rac1, respectively.

Microscopic Analyses

Glass coverslips were coated overnight at 4°C with collagen I (50 μg/ml; Collagen Corp.) or laminin-1 purified from EHS tumor (20 μg/ml; provided by Hinda Kleinman, NIDR, Bethesda, MD) and then blocked with BSA (0.25% in RPMI). Cells were plated on these coverslips for 30–40 min, rinsed with PBS, fixed, and then permeabilized as described previously (O'Connor et al., 1998). For immunofluorescence, cells were incubated with 1 μg/ml of K20 (anti-β1) and anti-RhoA antibody diluted in block solution (3% BSA/1% normal donkey serum in PBS) for 30 min, rinsed four times with PBS, and then incubated for 30 min with a 1:400 dilution of anti-mouse IgG Cy2- and anti-rabbit IgG Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). Images of cells were captured digitally, analyzed, and lamellar area quantified as described previously (Rabinovitz and Mercurio, 1997; O'Connor et al., 1998).

Migration Assays

The lower compartments of Transwell chambers (6.5-mm diam, 8-μm pore size; Costar) were coated for 30 min with 15 μg/ml laminin-1 diluted in RPMI medium. RPMI/BSA was added to the lower chamber and cells (1×10^5) suspended in RPMI/BSA were added to the upper chamber. After incubating for 5 h at 37°C, cells were removed from the upper chamber with a cotton swab and cells that had migrated to the lower surface of the membrane were fixed, stained with crystal violet or for β-galactosidase (β-gal), and quantified as described previously (Shaw et al., 1997).

RhoA Activity

RhoA activity was assessed using the Rho-binding domain of Rhotekin as described (Ren et al., 1999). In brief, cells (3×10^6) were plated onto 60-mm dishes coated with LN-1 (20 μg/ml) or collagen I (50 μg/ml) for 30 min and extracted with RIPA buffer (50 mM Tris, pH 7.2, 500 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1% SDS, 10 mM $MgCl_2$, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 4 μg/ml aprotinin, and 2 mM PMSF). Alternatively, cells were incubated with 8 μg of anti-β1 mAb mc13 or anti-β4 rat mAb 439-9B for 30 min, rinsed, plated on 60-mm dishes coated with 50 μg of either anti-mouse or anti-rat IgG, respectively, for 30 min, and then extracted. After centrifugation at 14,000 *g* for 3 min, the extracts were incubated for 45 min at 4°C with glutathione beads (Pharmacia Biotech) coupled with bacterially expressed GST–RBD (Rho-binding domain of Rhotekin) fusion protein (provided by Martin Schwartz, Scripps Research Institute, La Jolla, CA), and then washed three times with Tris buffer, pH 7.2, containing 1% Triton X-100, 150 mM NaCl, and 10 mM $MgCl_2$. The RhoA content in these samples was determined by immunoblotting samples using rabbit anti-RhoA antibody.

Results

Clone A colon carcinoma cells develop fan-shaped lamellae and exhibit random migration when plated on laminin-1, processes that are dependent on both the α6β4 and β1 integrins. In contrast, the β1 integrin-mediated adhesion and spreading of these cells on collagen I does not induce significant lamellae formation or migration (Rabinovitz and Mercurio, 1997; Shaw et al., 1997). To examine the hypothesis that RhoA functions in α6β4-dependent lamellae formation, clone A cells were cotransfected with a GFP construct and either a dominant negative RhoA (N19RhoA) or a control vector. Subsequently, the cells were plated onto laminin-1 and examined by phase-contrast microscopy. Clone A cells that expressed the control vector developed large lamellae with ruffled edges (Fig. 1 A). In contrast, cells that expressed N19RhoA developed only a few small, fragmented lamellae that were devoid of membrane ruffles (Fig. 1 B). Quantitative analysis of these images revealed that expression of N19RhoA reduced lamellar area by 80% in comparison to cells that expressed the control vector (Fig. 1 D). Interestingly, expression of a GST-tagged, dominant negative Rac1 (N17Rac1) did not inhibit either lamellae formation or membrane ruffling in clone A cells (Fig. 1, C and D), although this construct has been shown to inhibit p70 S6 kinase (Chou and Blenis, 1996) and invasion (Shaw et al., 1997).

Expression of N19RhoA inhibited the migration of clone A cells on laminin-1 by 70% (Fig. 2 A). In contrast, expression of N17Rac did not inhibit the migration of clone A cells (Fig. 2 A), although it did inhibit the migration of 3T3 cells by 85% (data not shown). Importantly, expression of N19RhoA had only a modest effect on cell spreading because cells expressing N19RhoA plated on collagen–I spread to ~80% of the surface area occupied by control cells (Fig. 1 E). Expression of N19RhoA and N17Rac1 in clone A cells was confirmed by immunoblotting (Fig. 1, F and G).

Our observation that RhoA functions in lamellae for-

[1]*Abbreviations used in this paper:* β-gal, β-galactosidase; GFP, green fluorescent protein; IBMX, isobutylmethylxanthine; LPA, lysophosphatidic acid; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; RBD, Rho-binding domain of Rhotekin.

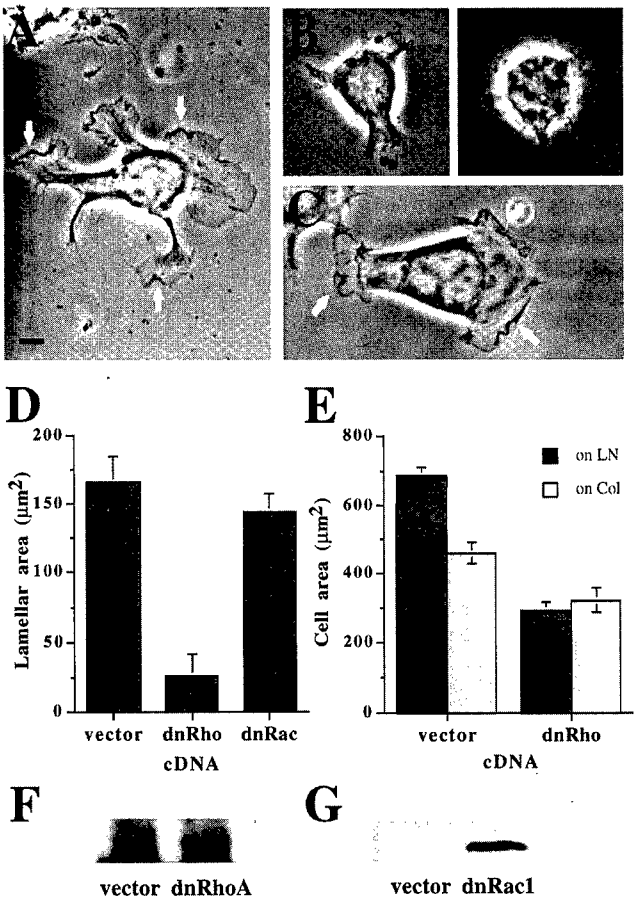

Figure 1. Dominant negative RhoA inhibits membrane ruffling and lamellae formation in clone A cells in response to laminin-1. Clone A cells were cotransfected with a GFP construct and either a control vector or a vector encoding N19RhoA or N17Rac as described in Materials and Methods. Cells were plated onto laminin-coated coverslips for 40 min, fixed, and assessed by phase-contrast microscopy. A–C, Phase-contrast microscopy of vector control (A), N19RhoA (B, two panels), or N17Rac (C) transfected cells. Note large lamellae and membrane ruffles in control and N17Rac transfected cells (open arrow in A and C), but not in cells that express N19RhoA (B). Representative GFP-positive cells are shown. D, Quantitative analysis of the lamellar area of transfected, GFP-positive cells was obtained by digital imaging. Lamellae are defined as broad, flat cellular protrusions rich in F-actin and devoid of membrane-bound vesicles. E, Quantitative analysis of total area covered by cells transfected with either vector control or N19RhoA when plated on laminin-1 (dark bars) or collagen I (light bars). Bars represent mean area ± SEM in which $n > 20$ (D, E). F and G, Transfected cells were extracted with RIPA buffer and either immunoprecipitated with HA-specific mAb and immunoblotted for RhoA (F), or concentrated using glutathione-Sepharose and immunoblotted for Rac1 (G). Representative blots are shown.

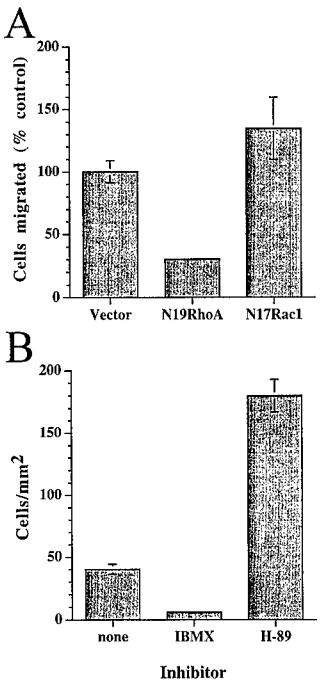

Figure 2. Effects of dominant negative RhoA (N19RhoA) and cAMP metabolism on laminin-1 stimulated migration. A, Clone A cells that had been cotransfected with a β-gal cDNA and either N19RhoA, N17Rac1, or control vector were assayed for migration on laminin-1 as described in Materials and Methods. Migration was scored as the relative number of β-gal staining cells migrated compared with the vector only control. Transfection rates were comparable. B, Clone A cells were left untreated or treated with 1 mM IBMX or 15 μM H-89 for 15 minutes and then assayed for laminin-1 mediated migration as described in Materials and Methods. Migration rates were reported as the number of cells migrated per mm². Bars represent mean ± SD from triplicate determinations.

mation and the migration of clone A cells, in conjunction with our previous finding that these events require the engagement of the α6β4 integrin (Rabinovitz and Mercurio, 1997), indicated that α6β4 may mediate the activation of RhoA. To assess RhoA activation, we used the RBD to capture GTP-bound RhoA from cell extracts (Ren et al., 1999). As shown in Fig. 3, the interaction of clone A cells with laminin-1, which requires α6β4, resulted in a significant amount of RhoA retained by RBD in comparison to the interaction of these cells with collagen I, which does not involve α6β4 directly. These experiments were performed with cells that had been attached to laminin for 30 min because membrane ruffling was most apparent at this time. Quantitative analysis of the results obtained in four independent experiments revealed a threefold greater increase in RhoA activation in cells plated on laminin-1 than in cells plated on collagen (Fig. 3 B). To establish the ability of α6β4 to activate RhoA more definitively, we used integrin-specific mAbs to cluster both α6β4 and β1 integrins. As shown in Fig. 3, C and D, clustering of α6β4 resulted in an approximate two- to threefold higher level of RhoA activity in comparison to cells maintained in suspension. Interestingly, clustering of β1 integrins actually decreased RhoA activation in comparison to cells maintained in suspension (Fig. 3), even though clone A cells express similar surface levels of both integrins (Lee et al., 1992). Similar results were obtained between 5 and 30 min of antibody clustering (data not shown).

The involvement of cAMP metabolism in migration, lamellae formation, and α6β4-mediated RhoA activation was investigated using both IBMX, a phosphodiesterase (PDE) inhibitor, and H-89, a cAMP-dependent protein kinase (PKA) inhibitor. IBMX treatment, which prevents cAMP breakdown, inhibited the migration of clone A cells on laminin-1 almost completely (Fig. 2 B). In contrast, inhibition of PKA with H-89 increased the rate of migration by fourfold (Fig. 2 B). Together, these data indicate that cAMP inhibits or "gates" carcinoma migration and lamellae formation, in agreement with our previous findings (O'Connor et al., 1998). To establish the involvement of cAMP metabolism in the α6β4-mediated activation of

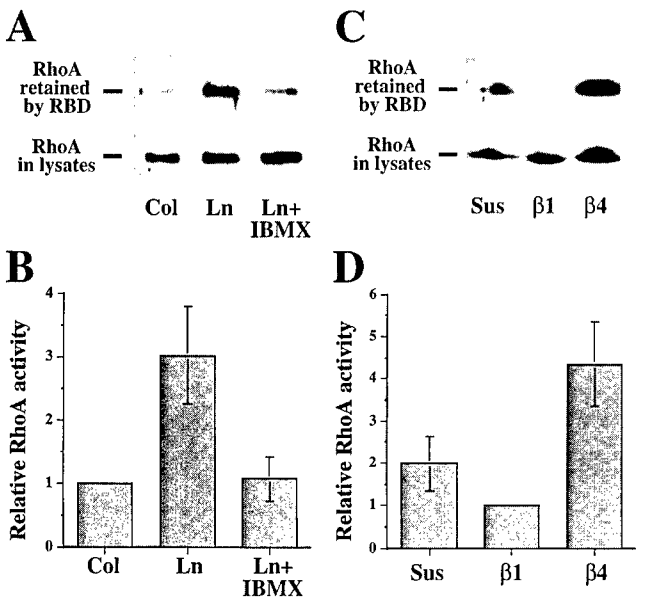

Figure 3. Engagement of the α6β4 integrin by either laminin-1 or antibody-mediated clustering activates RhoA. A and B, Clone A cells were plated on either collagen or laminin for 30 min or pretreated with 1 mM IBMX for 15 min and then plated on laminin for 30 min. Cell extracts were assayed for Rhotekin binding activity as described in Materials and Methods. C and D, Cells were either left in suspension (sus) or clustered with either β1- or β4-specific antibodies for 30 min as described in Materials and Methods. Cell extracts were assessed for RhoA activity by RBD binding. For these experiments, the total RhoA bound to the RBD (top panels in A and C) was normalized to the RhoA content of cell extracts (bottom panels in A and C). A and C, Representative immunoblots from these experiments are shown. B and D, Quantitative analysis of the results obtained by densitometry is provided. Bars represent mean of four separate experiments ± SEM.

RhoA, we used IBMX in the RBD assay. As shown in Fig. 3 A, pretreatment of clone A cells with IBMX before plating on laminin-1 reduced the level of RhoA activation to that observed in cells plated on collagen. Importantly, IBMX did not inhibit either cell adhesion or spreading (Fig. 4, C and E). Similar results were obtained with integrin clustering (data not shown). These observations implicate cAMP metabolism in the α6β4-mediated activation of RhoA.

The data reported here raise the possibility that α6β4 influences RhoA localization because activation of RhoA is thought to involve its translocation to membranes (Bokoch et al., 1994). To address this issue, clone A cells plated on either laminin-1 or collagen I were immunostained with a RhoA-specific antibody, as well as a β1-integrin–specific antibody to mark membranes. In cells plated on collagen I, RhoA immunostaining was confined largely to the cytosol and it was distinct from the β1-integrin staining of the plasma membrane (Fig. 4 A). In contrast, the α6β4-dependent interaction of clone A cells with laminin-1 resulted in the translocation of RhoA to membrane ruffles at the edges of lamellae where it colocalized with β1 integrin staining (Fig. 4 B). However, RhoA did not colocalize with β1 integrins on the plasma membrane along the cell body (Fig. 4 B). To assess the influence of cAMP metabolism on RhoA localization, clone A cells were pretreated with either IBMX or H-89 before plating on laminin-1. Inhibition of PDE activity with IBMX dramatically inhibited membrane ruffling and abolished RhoA localization in the few ruffles that persisted after IBMX treatment (Fig. 4, C and E). Conversely, inhibition of cAMP-dependent PKA with H-89 resulted in an apparent increase in membrane ruffling and RhoA localization in membrane ruffles (Fig. 4, D and F).

Discussion

Recently, we established that the α6β4 integrin stimulates the migration of carcinoma cells and enhances the formation of actin-rich protrusions, including lamellae and membrane ruffles (Shaw et al., 1997; Rabinovitz and Mercurio, 1997; O'Connor et al., 1998; Rabinovitz et al., 1999). In this study, we advance our understanding of the mechanism by which α6β4 functions in these dynamic processes by demonstrating that ligation of α6β4 with either antibody or laminin-1 results in the activation of RhoA and its translocation from the cytosol to membrane ruffles at the leading edges of migrating carcinoma cells. Importantly, we also provide evidence that the α6β4-mediated activation of RhoA is necessary for lamellae formation, membrane ruffling, and migration. Furthermore, we establish that these events are regulated by cAMP metabolism and that they can occur independently of Rac1 involvement.

Our findings strengthen the evidence that integrins can participate in the activation of RhoA. Much of the evidence supporting integrin activation of RhoA had been based largely on the observation that integrin activation leads to the Rho-dependent formation of stress fibers and focal adhesions (Ren et al., 1999; Schoenwaelder and Burridge, 1999). Recently, the development of a biochemical assay for RhoA activation using the ability of GTP-bound RhoA to associate with the Rho-binding domain of Rhotekin has enabled a more rigorous and sensitive assessment of the mechanism of RhoA activation (Ren et al., 1999). Using this assay, cell attachment to fibronectin was shown to activate RhoA and that the level of activation was augmented by serum or lysophosphatidic acid (LPA). In our study, we extend this observation by providing evidence that a specific integrin, α6β4, can activate RhoA, as assessed by both Rhotekin binding and translocation to membrane ruffles. An interesting and unexpected finding obtained in our study is that the α6β4 integrin is a more effective activator of RhoA than β1 integrins in clone A cells. In fact, antibody-mediated ligation of β1 integrins actually suppressed RhoA activation. Because we used carcinoma cells in our study, the potent activation of RhoA we observed in response to α6β4 ligation could have resulted from a cooperation of α6β4 with a secreted growth factor or activated oncogene. However, if cooperative signaling between integrins and such factors occurs in these cells, it is specific for α6β4 because clustering of β1 integrins did not activate RhoA.

Our findings implicate an important role for RhoA in the formation of membrane ruffles and lamellae. Specifically, the expression of N19RhoA in clone A cells attached to laminin resulted in the appearance of frag-

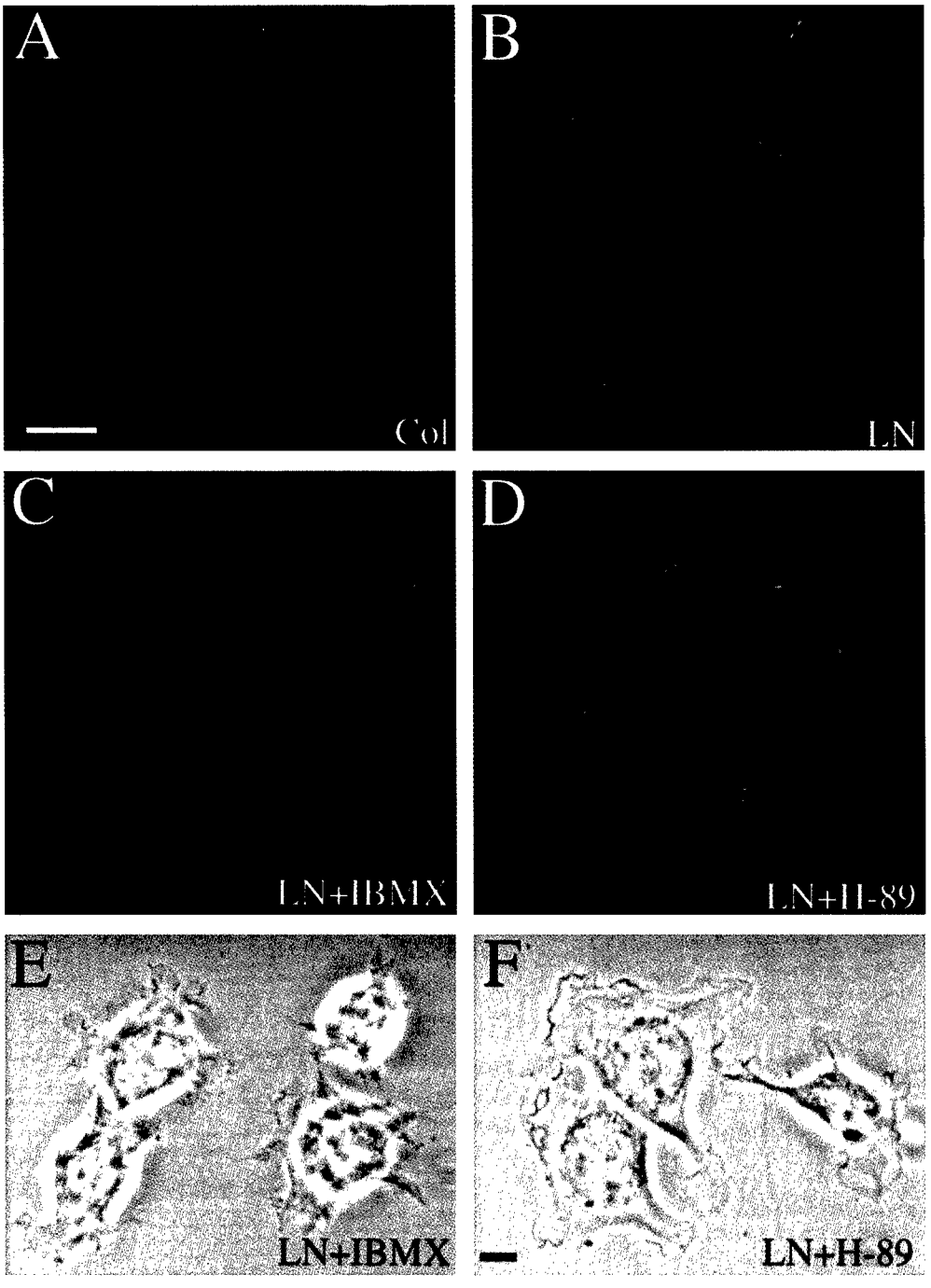

Figure 4. Laminin-1, but not collagen-I, promotes the colocalization of RhoA and β1 integrin in membrane ruffles in a cAMP-sensitive manner. A–D, Clone A cells were plated on either collagen I (A) or laminin-1 (B–D) for 30 min, fixed, and stained for both β1 integrin and RhoA using indirect immunofluorescence as described in Materials and Methods. To assess the impact of cAMP signaling on recruitment of RhoA to the plasma membrane, cells were pretreated with 1 mM IBMX (C) or 15 μM H-89 (D) for 15 min before plating cells on laminin-1. Images were captured digitally ~1 μm from the basal surface using a Bio-Rad confocal microscope. Red color represents RhoA; green, β1 integrin; yellow, β1 and RhoA colocalization. E and F, Phase-contrast micrographs of cells treated with IBMX (E) or H-89 (F) and plated on laminin depict the general impact of cAMP metabolism on membrane ruffling. Bars, 10 μm.

mented, immature lamellae and a loss of membrane ruffles. These results are of interest in light of recent reports that Rho kinase, a downstream effector of Rho, promotes membrane ruffling in epithelial-derived cells (Nishiyama et al., 1994; Fukata et al., 1999) by a mechanism that involves Rho kinase-mediated phosphorylation of adducin (Fukata et al., 1999). Moreover, both Rho and Rho kinase have been implicated in tumor cell invasion (Yoshioka et al., 1998; Itoh et al., 1999). Together, these findings along with our previous work that established the ability of α6β4 to promote carcinoma migration and invasion (Rabinovitz and Mercurio, 1997; Shaw et al., 1997; O'Connor et al., 1998), suggest that α6β4-mediated regulation of the Rho/Rho kinase pathway is an important component of carcinoma progression. It is also possible that the α6β4-mediated activation of RhoA contributes to migration and invasion by activating the adhesive functions of other integrins. RhoA can activate integrin-mediated adhesion in leukocytes (Laudanna et al., 1997) and is believed to participate in adhesion in other cell types (Nobes and Hall, 1999). Our observation that RhoA and β1 integrins colocalize in membrane ruffles in response to α6β4 ligation raises the possibility that RhoA influences the function of β1 integrins, which are essential for migration and invasion.

Interestingly, expression of the dominant negative

N17Rac in clone A cells had no inhibitory effect on either membrane ruffling, lamellae formation, or migration. Although it is well established that Rac functions in lamellipodia formation in fibroblasts (Hall, 1998) and in the migration of several cell types (e.g., see Keely et al., 1997; Shaw et al., 1997; Nobes and Hall, 1999), recent studies have highlighted the complexity of Rac involvement in these dynamic processes. For example, Rac activation can also inhibit migration by promoting cadherin-mediated cell–cell adhesion (Hordijk et al., 1997; Sander et al., 1999) and by downregulating Rho activity (Sander et al., 1999). Nonetheless, Rac activation stimulates membrane ruffling under conditions in which it also promotes cell–cell adhesion (Sander et al., 1999). Clone A cells, therefore, may represent the first example of a cell type in which both membrane ruffling and migration are Rac-independent.

Our results highlight the importance of cAMP metabolism in the activation and localization of RhoA. Our finding that cAMP inhibits RhoA activation and translocation to membrane ruffles is consistent with our previous report that linked the ability of α6β4 to promote carcinoma migration with its ability to alter cAMP metabolism (O'Connor et al., 1998). In addition, these results substantiate other studies that indicated an inhibitory effect of cAMP on RhoA activity (Lang et al., 1996; Laudanna et al., 1997; Dong et al., 1998). The basis for this inhibition may be the direct phosphorylation of RhoA by PKA (Lang et al., 1996). In this context, α6β4 may contribute to RhoA activation by increasing the activity of a cAMP-dependent PDE and subsequently reducing PKA activity, as we have suggested previously (O'Connor et al., 1998). However, the fact that we observed RhoA activation in response to antibody-mediated clustering of α6β4 suggests this integrin can be linked directly to RhoA activation. This direct activation of RhoA would permit α6β4 to augment pathways, such as LPA signaling, that involve RhoA activation. In conclusion, the results reported here establish a specific integrin-mediated pathway of RhoA activation that is regulated by cAMP and functions in lamellae formation and migration.

We thank Steve Akiyama, Rita Falcioni, Hynda Kleinman, Martin Schwartz, and Alex Toker for reagents. We also acknowledge Isaac Rabinovitz and Robin Bachelder for helpful discussions.

This work was supported by the United States Army Medical Research and Materiel Command Grants DAMD17-98-1-8033 (to K.L. O'Connor) and DAMD17-96-1-6199 (to A.M. Mercurio), as well as National Institutes of Health Grant CA80789 (to A.M. Mercurio) and the Harvard Digestive Diseases Center.

Submitted: 18 October 1999
Revised: 17 December 1999
Accepted: 20 December 1999

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Report

Integrin-mediated Stimulation of Protein Kinase A Regulates Rac and Promotes the Chemotactic Migration of Carcinoma Cells

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Running Title: Protein kinase A regulation of Rac
Key Words: cytoskeleton
chemotaxis
cyclic AMP
phosphodiesterase
lysophosphatidic acid
Total Characters: 19,683

Abbreviations used in this paper are: $[cAMP]_i$, intracellular concentration of cyclic AMP; IBMX, isobutylmethylxanthine; LPA, lysophosphatidic acid; MHC, major histocompatibility complex; PKA, cAMP-dependent protein kinase; PKI, protein kinase A inhibitor; PBD, Rac binding domain of Pak1; PDE, phosphodiesterase.

Abstract:

Increases in the intracellular concentration of cAMP ($[cAMP]_i$) stimulate the activity of protein kinase A (PKA) and impede the migration of carcinoma cells by a mechanism that involves PKA phosphorylation of Rho A (O'Connor et al, *J. Cell Biol.* 148:253-258; Lang et al., *EMBO J.* 15:510-519). Phosphodiesterases (PDEs) are necessary for migration because they prevent this cAMP inhibition of RhoA (O'Connor et al, *J. Cell Biol.* 148:253-258). We report here that the chemotactic migration of breast carcinoma cells involves not only PDE activity but also a PKA-dependent signaling event involving Rac1. Importantly, we identify β1 integrin signaling as a stimulus of PKA activity in migrating cells and demonstrate that the activation of Rac by β1 integrins requires PKA activity. A novel implication of these findings is that PKA can differentially regulate the activity and function of Rac and Rho and it may mediate a spatial and temporal regulation of these GTPases during migration.

Chemotactic migration is essential for embryonic development, tissue homeostasis and the immune response (Horwitz and Parsons, 2000). It is also a major factor in the pathogenesis of many human diseases including cancer (Stetler-Stevenson, et al., 1993). This complex process involves dynamic and coordinated interactions among integrins, chemoattractant receptors and the actin cytoskeleton that result in actin polymerization at the leading edge and contraction of actin bundles within the cell body to promote translocation. These dynamic changes in the actin cytoskeleton are initiated by the engagement of chemoattractant and integrin receptors on the cell surface with their respective ligands. Such interactions trigger cascades of signaling events that result in the remodeling of the actin cytoskeleton and consequent directed movement (Condeelis, 1993). To understand chemotactic migration at a mechanistic level, these signaling events need to be defined and linked to both cell surface receptors and actin dynamics. Members of the Rho family of GTPases including Rho, Rac and cdc42, in particular, are considered to be key signaling intermediates for cell migration (Horwitz and Parsons, 2000; Keely, et al., 1997; Sander, et al., 1999; Sander, et al., 1998; Shaw, et al., 1997). These GTPases are activated by signaling pathways initiated at the cell surface and, in their activated or GTP-bound state, they stimulate downstream effectors that regulate actin polymerization and actin-myosin contraction (Horwitz and Parsons, 2000; Tapon and Hall, 1998). Despite considerable progress in understanding the function of the Rho GTPases, the mechanisms by which their activity is regulated by cell surface receptors and how this regulation relates to cell migration are understood poorly.

Recent studies have highlighted an important role for cAMP metabolism in the chemotactic migration of carcinoma cells and in the regulation of Rho A function. Specifically, we found that cAMP-specific phosphodiesterases (PDEs), by lowering cAMP levels, facilitate chemotactic migration, as well as lamellae formation (O'Connor, et al., 1998). Subsequent studies revealed that cAMP inhibits RhoA activity, which is required for lamellae formation and migration (O'Connor, et al., 2000). The finding that cAMP inhibits RhoA function is consistent

with previous reports demonstrating that PKA-mediated phosphorylation of Rho inhibits its function (Dong, et al., 1998; Lang, et al., 1996). In fact, based on these observations, the current study was initiated to test the hypothesis that PKA activity inhibits chemotaxis. Surprisingly, we observed that chemotactic migration requires PKA activity, as well as PDE activity. In other terms, our results infer that chemotaxis is controlled by cAMP-dependent, as well as cAMP-inhibitory signaling mechanisms. We provide an explanation for this paradox by demonstrating that PKA is required for Rac activation by β1 integrins, a function that contrasts with its inhibition of RhoA. An important implication of these findings is that localized fluctuations in the $[cAMP]_i$ may provide a spatial and temporal regulation of PKA activity that influences Rac and RhoA function.

Materials and Methods:

Cells: MDA-MB-435 human breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository at Georgetown University. Cells were cultured in Dulbecco's-modified Eagle's medium (DMEM) with 10% fetal calf serum plus 1% L-glutamine, 1% penicillin, and 1% streptomycin (GIBCO-BRL, Gaithersburg, MD). For each experiment, subconfluent cell cultures were harvested with trypsin and rinsed with either DMEM or RPMI medium containing 250μg/ml heat-inactivated BSA (DMEM/BSA and RPMI/BSA, respectively).

Migration assays: Chemotaxis assays were performed as described previously (O'Connor, et al., 1998). Briefly, Transwell chamber membranes (6.5mm diameter, 8μm pore size; Costar) were coated with 15μg/ml collagen I (trademark Vitrogen, Collagen Biomaterials). Lysophosphatidic acid (LPA; 100nM; Sigma) was added to the lower chambers. Cells ($5x10^4$) were added to the upper chamber and allowed to migrate for 4hrs at 37°C. Cells that had not migrated were removed from the upper chamber with a cotton swab. The remaining cells were fixed, stained, and counted as described previously (O'Connor, et al., 1998). In some experiments, the following pharmacological inhibitors were added to the cell suspensions for 30 min. before cells were added to wells: H-89; IBMX and a myristoylated peptide derivative of PKI (Calbiochem).

The involvement of Rac1 chemotactic migration was assessed using a dominant negative construct (N17Rac1). Briefly, cells were electoporated (O'Connor, et al., 2000) with 1μg of pCS2-(n)β-gal and 4μg of either control vector or GST-tagged N17Rac1 construct (obtained from Margaret Chou, University of Pennsylvania) at 250V and 500μFd. Cells were then cultured in growth medium containing 0.05% sodium butyrate for 16hrs and assayed for chemotactic migration 48hrs after the initial transfection as described above. N17Rac1 expression was confirmed by concentrating extracts of transfected cells with glutathione-coupled Sepharose 4B beads (Pharmacia) and immunoblotting for Rac1 (mouse mAb; Transduction Laboratories).

Rac activity assays: Rac activity assays were based on established protocols (Benard, et al., 1999; Sander, et al., 1998). For these experiments, serum starved cells (3×10^6) were treated with 8μg of anti-integrin β1 mouse mAb MC13 (obtained from Steve Akiyama, National Institutes of

Health, Research Triangle Park, NC) for 30 min. at room temperature. Cells were then pelleted, resuspended in RPMI/BSA and either plated onto goat anti-mouse IgG (50μg; Jackson Immunochemicals) coated 60mm dishes or left in suspension. Cells were incubated at 37°C for the indicated time periods and then harvested with 50mM Tris, pH 7.4, 100mM NaCl, 1% NP-40, 10% glycerol, 2mM $MgCl_2$ 10μg/ml leupeptin, 10μg/ml aprotinin, 1μg/ml pepstatin, 1mM PMSF. Cell extracts were cleared by centrifugation and 10% of the total volume was used for assessment of total Rac content. The remaining extract was combined with 2 volumes of binding buffer (25mM Tris, pH 7.5, 1mM DTT, 40mM NaCl, 30mM $MgCl_2$, 0.5% NP-40) and bacterially produced Rac/cdc42 binding domain of Pak (PBD)-GST fusion protein (obtained from Rick Cerione, Cornell University, ref. Benard, et al., 1999) coupled to glutathione beads and then incubated for 45 min. at 4°C. Beads were then rinsed three times with binding buffer and eluted in 2X Laemmli sample buffer. Aliquots of both total cell extracts and the eluents from the PBD beads were immunoblotted for Rac1.

To assess the impact of LPA on Rac activation, cells were plated onto collagen-coated dishes in RPMI/BSA and allowed to attach and spread for 2-4 hours. Either the β1 integrin-specific mAb MC13 or control IgG was added to the dishes for 10 min. prior to stimulation with LPA for 10 min. Subsequently, cell extracts were assessed for Rac activity as described above.

PKA assays: For the PKA assays, cells were subjected to antibody mediated clustering of β1 integrins or MHC (using anti-HLA class I mAb, Sigma) as described above and harvested with a 50mM Tris buffer, pH 7.5 containing 5mM EDTA, 50mM NaF, 1mM sodium pyrophosphate and protease inhibitors. Cell extracts were sonicated and debris removed by centrifugation. Extracts were incubated for 5 min. at 30°C in reaction buffer (final concentration: 50mM Tris, pH 7.5, 10mM $MgCl_2$, 100μM ATP, 4nmol [γ-^{32}P] ATP, 0.25mg/ml BSA and 50μM Kemptide; Life Technologies) alone (control) or in the presence of either 1μM PKI peptide (background), 10μM cAMP (total PKA activity) or PKI plus cAMP (total activity background). Samples were assayed in triplicate for each condition and quantified on a scintillation counter. PKI-inhibitable kinase activity was calculated and the data were reported as percent total PKA activity.

Results and Discussion

Chemotactic migration requires both PKA and PDE activities: PKA is activated in response to cAMP generated by adenyl cyclases (Taussig and Gilman, 1995). We hypothesized that PKA is inhibitory for chemotactic migration based on our previous findings that chemotaxis requires PDE activity and that it is promoted by increasing PDE activity and reducing intracellular cAMP levels (O'Connor, et al., 1998). To test this hypothesis, we assessed the chemotactic migration of MDA-MB-435 breast carcinoma cells toward LPA in the presence or absence of the PKA-specific inhibitor, H-89. Surprisingly, treatment of these cells with concentrations of H-89 known to be specific for PKA *in vivo* (Chijiwa, et al., 1990) resulted in a concentration-dependent inhibition of migration (Fig. 1A). The inhibitory effects of H-89 were confirmed using a myristoylated, cell permeable peptide derivative of the naturally occurring PKA Inhibitor (PKI; Fig. 1B). Together, these data emphasize the importance of PKA in chemotactic migration. As expected from our previous studies (O'Connor, et al., 1998), the PDE inhibitor IBMX also blocked the LPA-stimulated chemotaxis of MDA-435 cells (Figure 1A and O'Connor, et al., 1998). Moreover, the combined inhibitory effects of IBMX and H-89 treatment on migration were additive indicating that the chemotactic migration of these carcinoma cells is regulated by cAMP-dependent as well as cAMP-inhibitory signaling mechanisms. These findings suggest the possibility that localized fluctuations or gradients in $[cAMP]_i$ may be necessary for chemotactic migration.

β1 integrin signaling stimulates PKA activity: Given that both β1 integrins (O'Connor, et al., 1998) and PKA (Fig. 1) are required for chemotactic migration on collagen, we tested the possibility that β1 integrin signaling stimulates PKA activation. To address this hypothesis, β1 integrins were clustered with a specific mAb for varying periods of time and cell extracts were assayed for PKA activity. As shown in Figure 2A, mAb-mediated clustering of β1 integrins resulted in a rapid, three-fold activation of PKA that decayed to baseline levels by 30 min. The specificity of this activation is indicated by the finding that clustering MHC class 1 molecules, which are expressed on the surface of MDA-435 cells at levels comparable to β1 integrins (data

not shown), did not stimulate PKA activity (Figure 2B). These data suggest that β1 integrin signaling is a potential source of PKA activation required for chemotactic migration. The mechanism of PKA activation by β1 integrin signaling likely involves the stimulation of adenyl cyclase activity and a localized rise in $[cAMP]_i$ that increases PKA activity. Surprisingly, the regulation of PKA activity by integrin signaling has not been explored previously and our findings should provide an impetus for future work in this area.

Rac activation by β1 integrins requires PKA: The small GTPase Rac is necessary for the migration and invasion of carcinoma cells (Keely, et al., 1997; Sander, et al., 1998; Shaw, et al., 1997) including MDA-MB-435 (Fig. 3A and Shaw, et al., 1997). Based on the findings that Rac and Rho often differ in their function and regulation and that cAMP impedes Rho function, we hypothesized that PKA activity is required for Rac activation in MDA-MB-435 cells.

To examine the involvement of PKA in Rac activation, we initially analyzed the stimuli that activate Rac in response to LPA. In response to a chemoattractant such as LPA, new β1 integrin-mediated contacts are formed at the leading edge to generate the traction necessary for migration (Felsenfeld, et al., 1996; Horwitz and Parsons, 2000). Thus, both LPA and β1 integrin signaling are possible stimuli for Rac activation. Using the Pak binding domain, we assessed the ability of both stimuli to activate Rac. As shown in Fig. 3C, LPA stimulation of MDA-435 cells adherent to collagen increased Rac activation. This activation was blocked, however, by the addition of a β1 integrin-specific mAb (Fig. 3C) indicating that the LPA stimulation of Rac is dependent on β1 integrins. The addition of this mAb did not perturb the attachment of the cells to collagen (data not shown). Most likely, it prevented the formation of new β1 integrins contacts in response to LPA. A more direct confirmation of β1 integrin activation of Rac was obtained by antibody-mediated clustering of β1 integrins. Indeed, clustering of β1 integrins induced a time-dependent activation of Rac that was maximal at 30 min. (Fig. 4A). Importantly, inhibition of PKA activity with either H-89 (Fig. 4B) or PKI (Fig. 4C) prevented Rac activation by β1 clustering. These PKA inhibitors did not interfere with cell attachment to the Ab-coated wells

(data not shown). Together, these results indicate that Rac activation in response to clustering of β1 integrins requires PKA activity.

The finding that PKA activity is needed for integrin-mediated activation of Rac in breast carcinoma cells extends the importance of cAMP signaling in both chemotactic migration and the regulation of Rho family GTPases. Indeed, it appears that chemotactic migration involves both cAMP synthesis by adenyl cyclases and cAMP catabolism by phosphodiesterases. We suggest that cAMP synthesis is needed for PKA-mediated stimulation of Rac activation as shown here. In contrast, the inhibition of Rho activation by PKA-phosphorylation is released by cAMP catabolism and the consequent suppression of PKA activity (O'Connor, et al., 2000). Unlike Rho, the mechanism by which PKA stimulates Rac activation is unlikely to involve direct phosphorylation because Rac does not contain a consensus PKA phosphorylation site. For this reason, PKA may regulate Rac indirectly by modifying the function of molecules that control Rac activation.

Recent studies have indicated a 'reciprocal' relationship between Rac and Rho activation and have provided evidence that Rac can inhibit Rho activity (Sander, et al., 1999). Our findings suggest that PKA may contribute to the differential regulation of these GTPases. Increases in PKA activity would be expected to facilitate Rac activation and impede Rho activation, and *vice versa*. Given that cAMP regulates PKA activity, the argument can be made that 'microgradients' of the $[cAMP]_i$ within migrating cells differentially influence Rac and Rho function by regulating PKA activity. In support of this argument, localized gradients of the $[cAMP]_i$ have been implicated in the regulation of growth cone movement (Song, et al., 1997). Moreover, this argument implies spatial and temporal differences in the activation of Rac and Rho. Such differences could be manifested, for example, in the Rac-mediated lamellipodial protrusion and Rho-mediated contractility necessary for migration (Hall, 1998; Horwitz and Parsons, 2000). In summary, our findings highlight the importance of cAMP and PKA in the regulation of small GTPase function and migration and identify important venues for future work.

Acknowledgements:

We acknowledge Steve Akiyama and Rick Cerione for reagents. We also thank Don Senger and Judy Glavin for helpful discussions, and Bao-Kim Nguyen for technical assistance. This work was supported by the United States Army Medical Research and Materiel Command Grants DAMD17-98-1-8033 (K. L. O.) and DAMD17-96-1-6199 (A. M. M.) as well as National Institutes of Health Grant CA80789 (A. M. M.) and the Harvard Digestive Diseases Center.

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Figure Legends:

Figure 1. Chemotactic migration of MDA-MB-435 carcinoma cells requires both protein kinase A (PKA) and phosphodiesterase (PDE) activities. **(A)** MDA-MB-435 cells were treated with various concentrations of the PKA-specific inhibitor H-89 either alone (□) or in conjunction with 100μM (◪) or 500μM of the PDE inhibitor IBMX (■). Cells ($5x10^4$) were then assayed for chemotaxis toward 100nM LPA in the continued presence of these inhibitors using a Transwell chamber assay as described in the Materials and Methods. **(B)** Cells were treated with either 1μM of a myristoylated, cell permeable peptide derivative of PKI or 15μM of H-89 for 30 min. and then assayed for chemotaxis as described above. Values reported represent the mean number of cells migrated per mm^2 ± standard deviation obtained from triplicate determinations.

Figure 2. β1 integrin signaling activates PKA. **(A)** MDA-MB-435 cells were incubated with MC13, a β1 integrin-specific mouse mAb, for 30 min. Subsequently, the cells were added to tissue culture wells that had been coated with an anti-mouse IgG antibody. At the indicated times, cells were extracted and assayed for PKA activity as described in Materials and Methods. **(B)** Cells were maintained in suspension or incubated with either the β1 integrin mAb or an MHC-specific mAb and then added to tissue culture wells that had been coated with secondary antibody. After 5 min., cells were extracted and assayed for PKA activity. Values reported are the mean percent of total PKA activity ± standard deviation. Data are representative of four separate experiments.

Figure 3. **(A)** LPA-stimulated chemotaxis of MDA-435 cells requires Rac. Cells that had been co-transfected with a β-gal construct and either control vector or GST-tagged N17Rac were assayed for LPA stimulated chemotaxis as described in Fig. 1. Cells that had migrated were fixed and stained for β-gal. Transfected, β-gal positive cells were counted and reported as the relative number of cell migrated compared to the vector control. Bars represent mean ± standard

deviation of triplicate determinations. (**B**) Transgene expression was confirmed by precipitating GST-Rac1 from extracts of cells in (A) with glutathione beads and immunoblotting for Rac1. (**C**) LPA stimulation of Rac activation is dependent on β1 integrins. Cells were plated onto collagen-coated dishes and allowed to attach and spread. Subsequently, either the β1 integrin-specific mAb MC13 or a control IgG was added to the cells for 10 min. and the cells were the stimulated with 100nM LPA for an additional 10 min. Cell extracts were assessed for Rac activity using the Pak binding domain (PBD) assay as described in Material and Methods. Representative experiments are shown.

Figure 4. Rac activation by β1 integrins requires PKA. (**A, B**) Antibody-mediated clustering of β1 integrins was performed as described in Fig. 2 either in the presence (**A**) or absence (**B**) of 15μM H-89 for times indicated. Cell extracts were assayed for Rac activation using the PBD assay as described in the Materials and Methods. (**C**) Antibody-mediated clustering of β1 integrins was performed in either the presence or absence of the PKI peptide 30 min. and cell extracts were assayed for Rac activity as described above. Upper panels, Rac bound to the PBD; Lower panels, Total Rac expressed in cell extracts.

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Figure 1

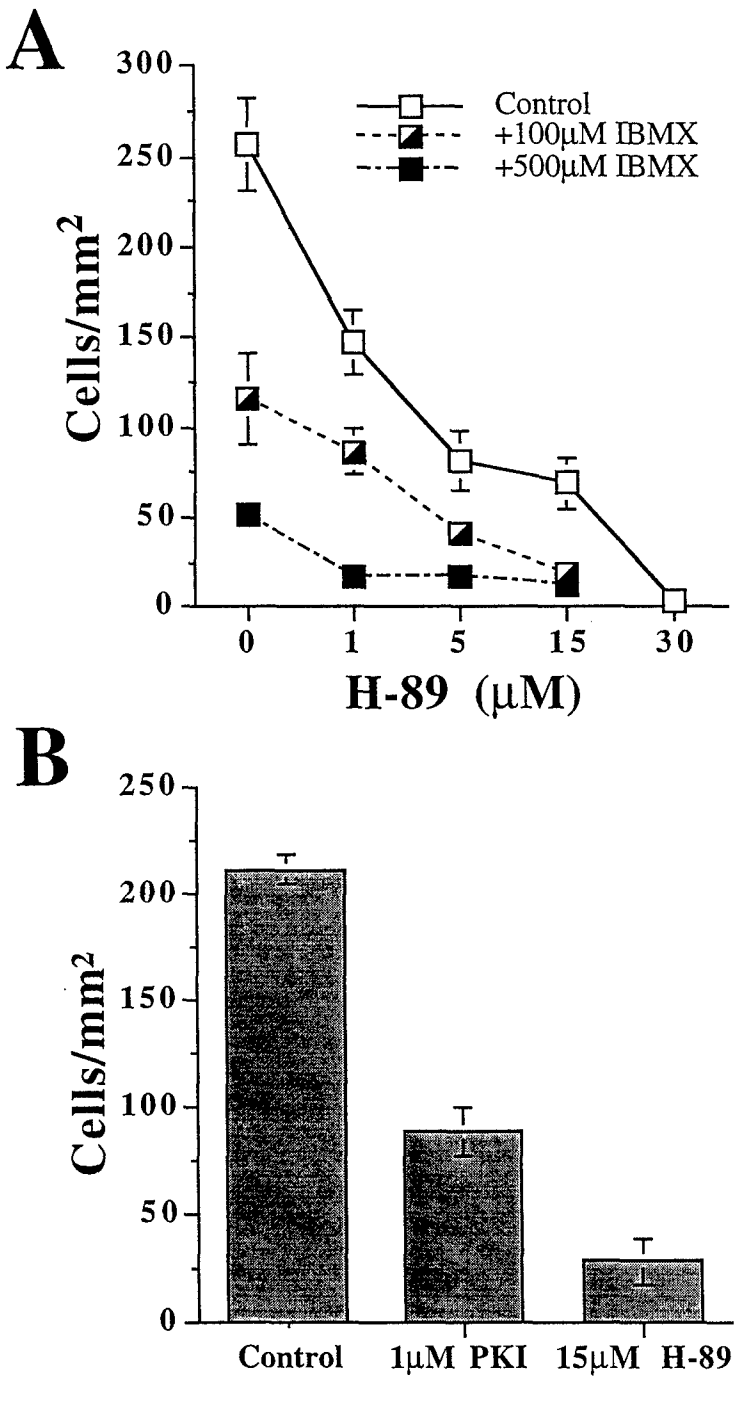

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Figure 2

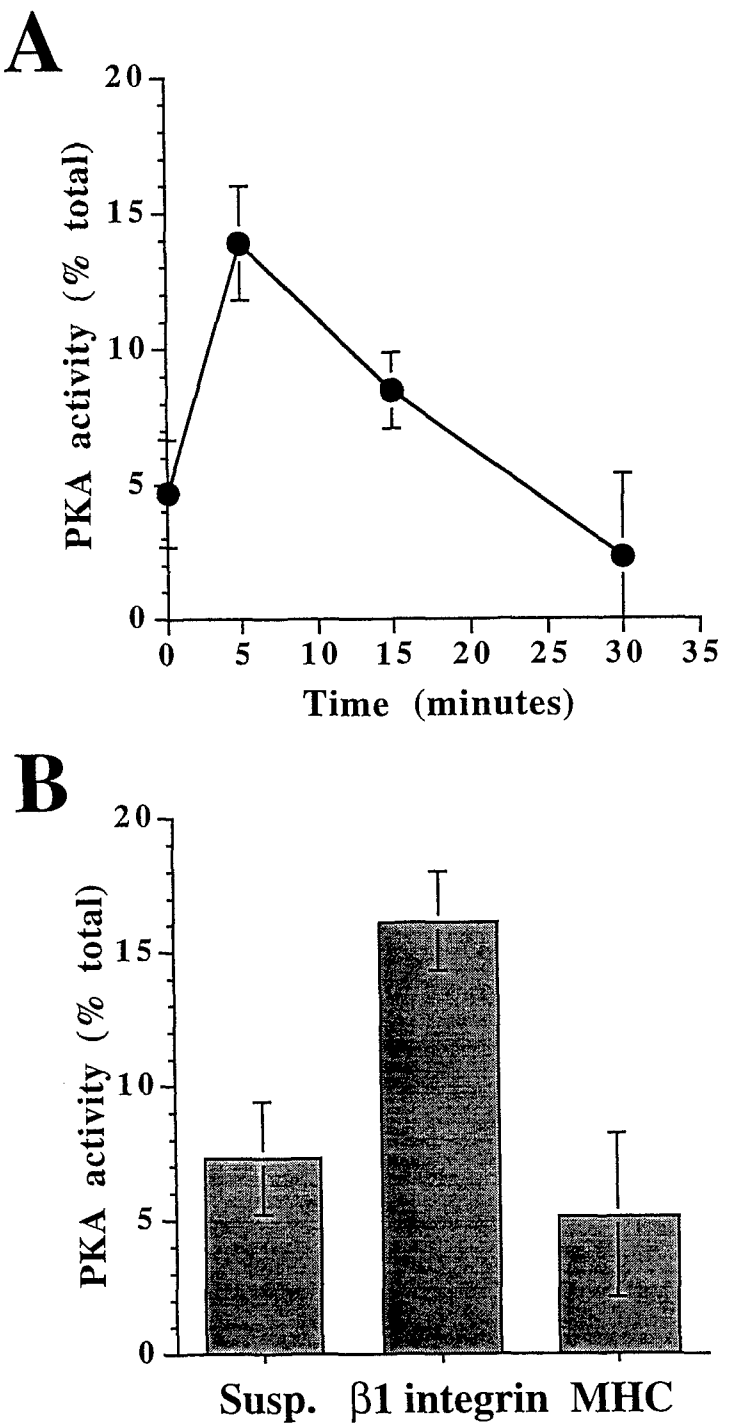

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Figure 3

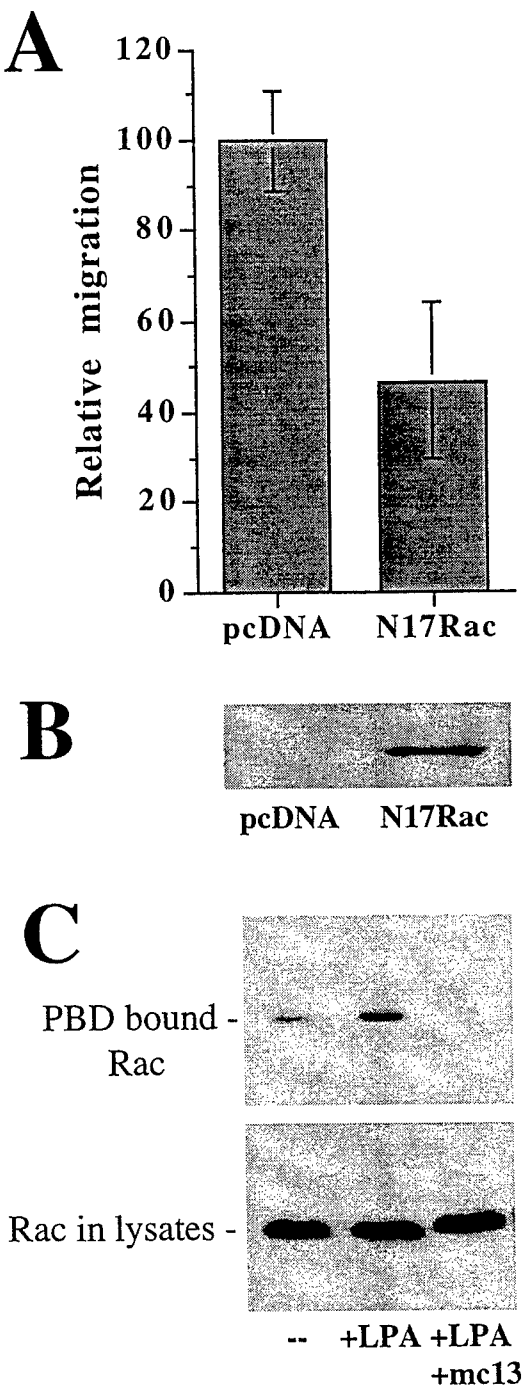
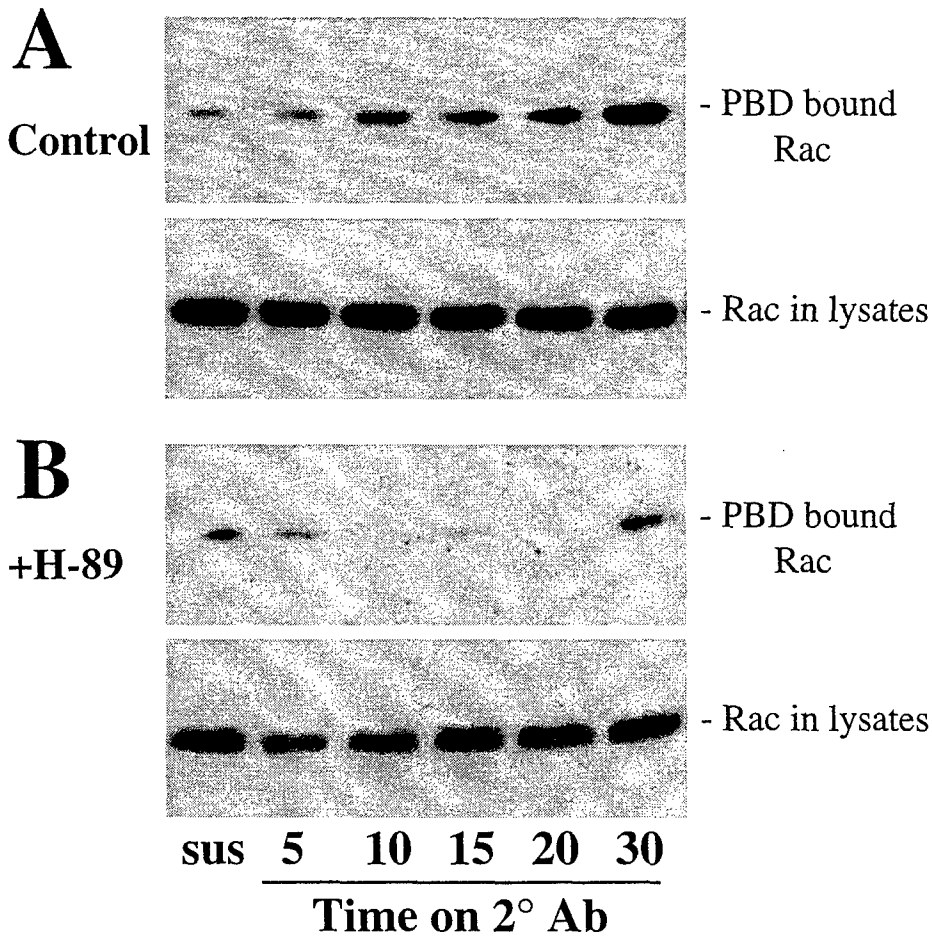
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Figure 4